MECHANISMS UNDERLYING THE ATTENUATION OF THE NEUROENDOCRINE RESPONSE OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS TO ACUTE STRESS IN THE LATE PREGNANT RAT

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Declaration

The studies presented in this thesis were carried out in the Department of Physiology, Medical School and the Department of Molecular Endocrinology, Molecular Medicine Centre, University of Edinburgh and at the Max Planck Institute of Psychiatry, Munich, Germany.

I declare that the studies presented in this thesis are the result of my own work and collaborations with others are indicated below.

i) The *in vitro* cAMP accumulation measurements from anterior pituitary segments and dispersed cells were accomplished with the help of Dr Mike Shipston.

ii) The CRF receptor autoradiography was carried out under the watchful eye of Gudrun Liebsch.

iii) The *in vivo* glycyrrhetinic acid experiment could not been completed without Dr Inga Neumann and Sandra Wigger.

This work has not been submitted for any other degree or at any other unversity.

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Abstract

Activation of the hypothalamic-pituitary-adrenal (HPA) axis by stressors results in the secretion of glucocorticoids, which contribute to the maintenance of homeostasis and autoregulate the activity of the axis via negative feedback mechanisms.

The aim of this PhD project was to examine whether HPA axis responses to acute stress were different during pregnancy and to elucidate possible mechanisms underlying these changes. We found that on day 21 of pregnancy the peak plasma adrenocorticotrophic hormone (ACTH) and corticosterone secretory responses to 20 min of restraint stress were significantly reduced.

Using receptor autoradiography we found that between day 10 and day 21 of pregnancy there was a progressive decrease in the binding of corticotropin-releasing factor (CRF) to anterior pituitary sections. When we examined the responsiveness of dispersed anterior pituitary cells to CRF and measuring the accumulation of cAMP, we found that on day 21 of pregnancy the anterior pituitary was less responsive. Using *in situ* hybridisation histochemistry to investigate the basal expression of CRF and arginine vasopressin (AVP) genes in the parvocellular paraventricular nucleus (PVN), we demonstrated that on day 21 of pregnancy there was a significant reduction in CRF, but not AVP mRNA.

We examined whether there was an increase in the sensitivity of the HPA axis to the glucocorticoid negative feedback signal. Using *in situ* hybridisation histochemistry we measured the basal expression of the mRNAs for mineralocorticoid (MR) and glucocorticoid (GR) receptors in the PVN and hippocampus and found that neither mRNA was altered during pregnancy. We also measured *in vitro* the bioactivity of the glucocorticoid-metabolising enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in PVN, anterior pituitary and hippocampus and found that the enzyme activity increased almost 3-fold selectively in the PVN on day 21 of pregnancy: 11β-HSD1 may act as a reductase, enhancing feedback *in vivo*. We went on to examine the impact of inhibiting central 11β-HSD on the response to forced swim (FS) stress. However, we found that central inhibition this enzyme had no significant effect on the ACTH and corticosterone secretory

response. We pharmacologically adrenalectomized pregnant and virgin rats and compared their ACTH response to exogenous corticosterone and found no difference in the overall suppression of ACTH secretion, although on day 21 of pregnancy the rats were less sensitive to the feedback signal within the first 30 min following the corticosterone injection.

When we implanted intact virgin female rats with subcutaneous estrogen- and progesterone-containing capsules, which elevated circulating estrogen and progesterone levels to concentrations similar to those found in pregnancy, and then exposed them to FS stress, we demonstrated that neither of these ovarian hormones influenced the HPA axis responses to stress.

To examine whether central endogenous opioid peptides (EOP) influenced the hypothalamic neuropeptide-containing neurones of the HPA axis, we administered naloxone (nlx) and then exposed the animals to FS stress. We demonstrated that nlx in virgin animals caused a significant suppression of the HPA axis responses to FS stress while in day 21 pregnant rats it caused a small non-significant elevation. This result indicates that in virgin animals central EOP enhance the HPA axis stress response and this enhancement is lost during pregnancy.

Thus there is a suppression of the responses of the HPA axis to acute stress in late pregnancy. Adaptations at the anterior pituitary and central level of the axis that may contribute to this attenuation include desensitization of corticotropes through CRF receptor loss, and decreased production of CRF by PVN neurones.

Abbrevations

11β-HSD	11β-hydroxysteroid dehydrogenase
ACTH	adrenocorticotrophic hormone
Adx	adrenalectomy
Amino	aminogluthetimide
ATP	adenosine triphosphate
AVP	arginine vasopressin
BNST	bed nucleus stria terminalis
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBG	corticosterone binding globulin
cDNA	complimentary deoxyribonucleic acid
CRF	corticotrophin-releasing hormone
cRNA	complimentary ribonucleic acid
CTP	cytosine triphosphate
Depc	diethyl pyrocarbonate
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
GA	18β-glycyrrhetinic acid
GTP	guanosine triphosphate
HPB	hypophysial portal blood
HPA Axis	hypothalamic-pituitary adrenal axis
HPLC	high preformance liquid chromatography
IBMX	3-isobutyl-1 methylxanthine
i.c.v.	intracerebroventricular
LC	locus coeruleus
OT	oxytocin
ME	median eminence
Met	metyrapone
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NTS	nucleus of the solitary tract
PVN	paraventricular nucleus
s.c.	subcutaneous
TLC	thin layer chromatography

Publications from this thesis

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Chapter One

Introduction

1.1 The Stress Response

It is essential for life that the body maintains a constant internal environment or homeostasis when challenged by either intrinsic and extrinsic forces, termed *stressors* and this state of threatened homeostasis is often referred to as *stress*.

The body reacts to stress by the activation of a complex repertoire of responses commonly known as the 'fight or flight' response, first described by Walter Cannon back in the early 1900s. This adaptive mechanism involves a number of behavioural, autonomic and neuroendocrine changes, which are coordinated by the CNS which constantly receives information from brain centres, the periphery and the environment. Thus, the stress response involves an extremely complex, but efficient and flexible physiological network which coordinates the maintenance of homeostasis.

1.2 Hypothalamic-pituitary-adrenal (HPA) axis

A well-characterised neuroendocrine change that occurs in response to stress is the activation of the hypothalamic-pituitary-adrenal (HPA) axis (fig 1.1) which is a centrally-driven system culminating in the secretion of glucocorticoids from the cortex of the adrenal gland.

The hypothalamic paraventricular nucleus (PVN) is the central coordinator of the HPA axis response to stress and receives information via stimulus-specific pathways. The hypophysiotropic CRF-containing neurones located in the medial paravocellular region of the PVN receive a rich afferent innervation (Sawchenko & Swanson, 1985). Viscerosensory information from the oral, thoracic and abdominal cavities is conveyed by ascending catecholaminergic pathways from the brainstem, primarily from the nucleus of the solitary tract (NTS) and the adrenergic C2 cell groups (Cunningham & Sawchenko, 1983). Several limbic structures, including the

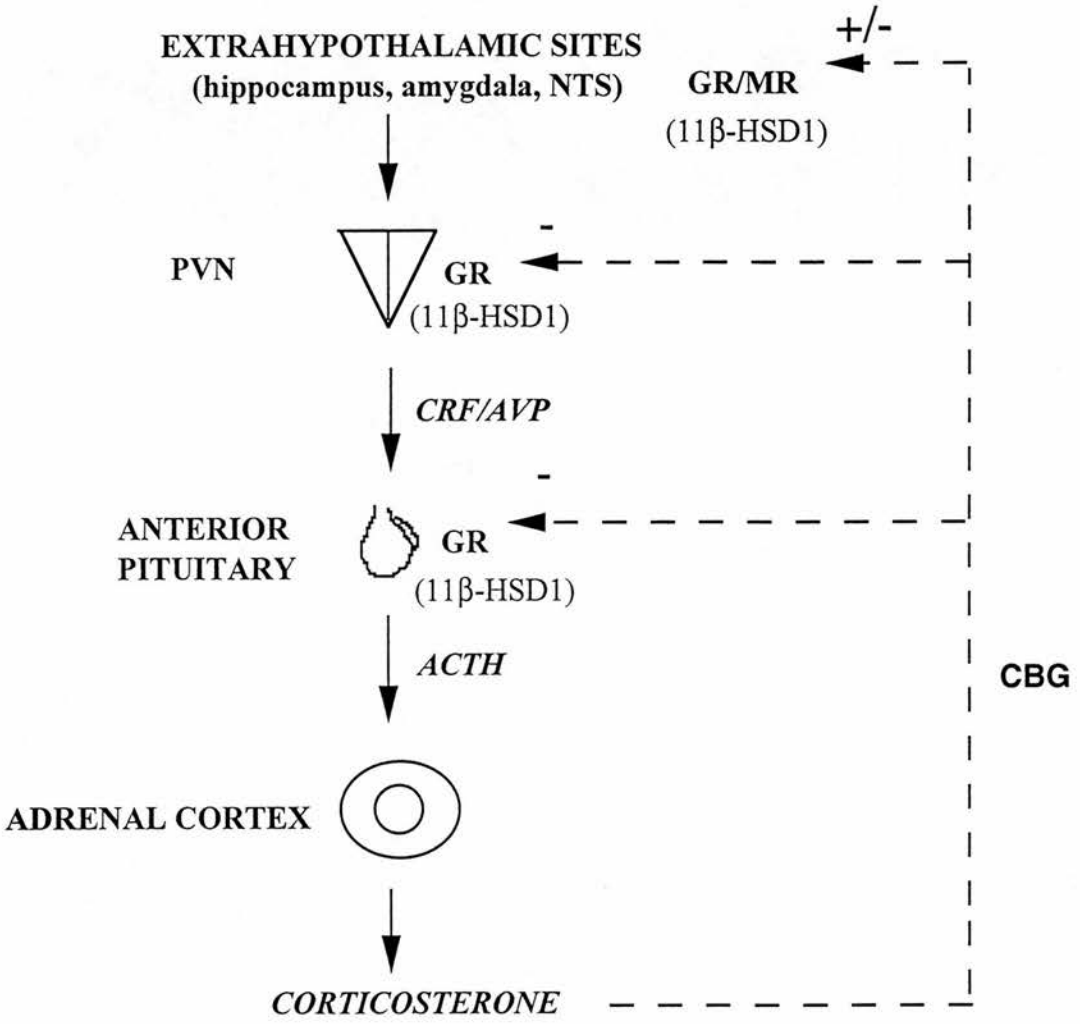


Fig 1.1: Schematic diagram of the hypothalamic-pituitary-adrenal (HPA) axis. **NTS** - nucleus of the solitary tract; **GR** - glucocorticoid receptor & **MR** - mineralocorticoid receptor; **11β-HSD1** - 11β-hydroxysteroid dehydrogenase type 1; **PVN** - paraventricular nucleus; **CRH** - corticotropin-releasing factor; **AVP** - arginine vasopressin; **ACTH** - adrenocorticotropin hormone; **CBG** - corticosterone binding globulin.

septum, amygdala and hippocampus are involved in conveying emotional stimuli indirectly via the bed nucleus of the stria terminalis (BNST), the lateral and medial septum, the fimbria and the preoptic area (Gray, 1991; Sawchenko & Swanson, 1983). Other stimuli, including humoral factors and availability of energy substrates may act through a series of interconnected cell groups comprising the lamina terminalis which lies partly outside the blood-brain barrier and is proposed to relay information carried by macromolecules and ions in the bloodstream (Gross, 1987).

Axons from the parvocellular CRF neurones extend to the external layer of the median eminence (ME) in the medial basal hypothalamus where they terminate on capillaries of the hypophysial portal blood (HBP) system. Upon stimulation, CRF and co-secretagogues, including arginine vasopressin (AVP), are released into the hypophyseal portal blood system where they act in a synergistic manner to release ACTH and other POMC-derived peptides from the anterior pituitary corticotrophs. ACTH is the key regulator of glucocorticoid secretion by the adrenal cortex as it increases the synthesis of glucocorticoids. Glucocorticoids are the final effectors of activation of the HPA axis. They are involved in the control of whole body homeostasis and the organism's response to stress. They play a key role in the regulation of the basal activity of the HPA axis and on the termination of the stress response by exerting negative feedback effects on the pituitary and specific brain regions to inhibit the secretion of CRF and AVP from hypothalamic neurones and ACTH from pituitary corticotrophs.

1.3 Paraventricular Nucleus

The paraventricular nucleus (PVN) comprises a group of cell bodies located in the hypothalamus of the forebrain. It is a complex structure with distinct magnocellular and parvocellular regions (Gurdjian, 1927). Retrograde neuronal tracing techniques (Swanson & Kuypers, 1980) indicated that this nucleus consists of at least eight distinct cell groups: three dense clusters of magnocellular neurones; anterior, medial and posterior, each containing neurones that express AVP or oxytocin: and an extensive parvocellular component that can be divided into five separate regions; anterior, medial, dorsal, lateral and periventricular.

Injection of two retrogradely transported fluorescent dyes, bisbenzimide-true blue and Evans blue-granular blue, into the posterior pituitary labelled the majority of cells in all three magnocellular subdivisions, with labelling of a few scattered cells in the parvocellular subdivisions (Swanson & Kuypers, 1980). However, injection of the dyes into the spinal cord or the dorsal vagal complex labels cells primarily in all the parvocellular regions, particularly in the anterior, medial and lateral areas (Swanson & Kuypers, 1980). These areas are rich in CRF-containing neurones involved in the control of autonomic functioning. However, the most widely studied population of PVN cell bodies are the CRF-containing neurones of the parvocellular medial subdivisions. Their major projection is to the external zone of the ME and they form the central effector of the HPA axis (Swanson & Kuypers, 1980).

1.3.1 Corticotropin-releasing factor (CRF)

In the late 1940s Harris (1948) proposed that the hypothalamus plays a key role in the regulation of adrenocorticotrophic hormone (ACTH) secretion from the anterior pituitary corticotroph cells. Then in 1955 Guillemin and Rosenburg and Saffran and Schally independently demonstrated the presence of factors in the hypothalamus that could increase the rate of ACTH secretion by the pituitary gland incubated *in vitro* or maintained in organ culture. However, the isolation and characterisation of the physiological corticotropin-releasing factor proved to be problematic. Therefore the existence of this elusive hypothalamic factor was not realised until the early 1980s when Wylie Vale and colleagues (Vale *et al.*, 1981) successfully isolated and characterised a 41 amino acid peptide from extracts of ovine hypothalamus. The compound exhibited potent ACTH-releasing activity on cultured anterior pituitary cells and was termed corticotropin-releasing factor (CRF). Subsequently, the primary structure of CRF from rat (rCRF) (Rivier *et al.*, 1983) was determined and found to differ from ovine CRF (oCRF) by only seven residues. The active portion of the molecule appears to reside in the carboxy-terminal (Aguilera *et al.*, 1983) since the absence of this portion prevents receptor binding. However, the presence of the amino terminal residues is necessary for full function since the

antagonist, α-helical CRF_{9-14}, which lacks the first eight amino acids, has a much weaker affinity for the CRF receptor than the native molecule.

The complementary DNAs (cDNA) encoding the CRF precursor have also been cloned (Furutani *et al.*, 1983; Jingami *et al.*, 1985b). This undergoes posttranslational proteolytic cleavage and amidation to produce the active protein. Analysis of the nucleotide sequence of the CRF gene demonstrated structural similarities between the human and rat (Thompson *et al.*, 1987): two exons separated by an intron with the nucleotide sequence encoding the entire CRF precursor located on the second exon.

1.3.2 Central distribution of CRF

The isolation of the CRF peptide and the subsequent cloning of the cDNA allowed immunohistochemical and hybridisation histochemical methods to be employed to study the distribution of the peptide and its mRNA.

Using polyclonal antibodies several investigators have found a widespread but selective distribution within the CNS (Swanson *et al.*, 1983; Merchenthaler, 1984). The largest population of CRF-immunoreactive cell bodies has been located in the dorsal aspect of the medial parvocellular region of the hypothalamic paraventricular nucleus. Their major projection is to the external zone of the median eminence which contains the primary capillary plexus of the hypothalamic-hypophysial portal blood system. (Antoni *et al.*, 1983; Liposits *et al.*, 1983). Other hypothalamic nuclei such as the supraoptic, suprachiasmatic, preoptic and arcuate nuclei (Daikoku *et al.*, 1984) also stain positively for CRF peptide.

A high density of CRF neurones is localised throughout the cortex; limbic system: amygdala and bed nucleus of the stria terminalis (BNST); and brainstem nuclei associated with autonomic functioning, locus coeruleus (LC) and parabrachial nucleus.

Several ascending CRF pathways have been reported. Within the dorsal vagal complex the nucleus of the solitary tract contains CRF cell bodies which project to the parabrachial nucleus (Herbert & Saper, 1990) and is proposed to be the main pathway for relaying somatosensory visceral information to the forebrain. The

parabrachial nucleus also contains CRF perikarya, whose terminal axonal field lies in the preoptic nucleus of the hypothalamus (Lind & Swanson, 1984). Following the isolation and cloning of the CRF gene the localisation of central sites of CRF synthesis were determined using both *in situ* hybridisation histochemistry and Northern blot analysis and in general the locations of CRF mRNA expression paralleled those of the immunohistochemical studies (Imaki *et al.*, 1989; Thompson *et al.*, 1987).

1.3.3 Corticotropin-releasing factor receptor

Several detailed autoradiographic studies have described the distribution of the CRF receptors in the pituitary and the CNS (De Souza *et al.*, 1984; Wynn *et al.*, 1984).

Autoradiographs of rodent pituitary (Aguilera *et al.* 1987) show specific binding sites for [^{125}I]-oCRF in the anterior and intermediate lobes with no specific binding evident in the posterior pituitary. Within the anterior pituitary the clustering of the binding sites corresponds to the distribution of corticotrophs with a more uniform distribution of binding occurring in the intermediate lobe of the pituitary, characteristic of the homogenous population of POMC-derived peptide producing cells in this lobe.

Receptor autoradiography in the brains of rodents and primates (De Souza *et al.*, 1985a; Millan *et al.*, 1986a) demonstrated the highest concentration of CRF-binding sites in brain regions involved in cognitive function, including the cerebral cortex, limbic areas involved in emotion and stress responses, including the amygdala, septum and hippocampus, and regions of the brainstem involved in the regulation of autonomic function, including the LC and NTS. The cerebellum, spinal cord and the olfactory bulb also displayed a high density of binding sites. The densities and localisation of CRF-binding sites correspond well with the distribution of CRF-immunoreactive fibres.

De Souza (1987) described in detail the binding characteristics of CRF to membrane homogenates from rat anterior pituitary and brain regions. He demonstrated that the binding was time-, temperature- and tissue concentration-

dependent, saturable and reversible. Scatchard analysis revealed a high-affinity binding (Kd = 0.2 nM) and a low affinity (Kd = 20 nM) form, thus fulfilling the criteria of a *bona fide* receptor.

The CRF receptor is positively coupled to the adenylate cyclase second messenger system via a guanine nucleotide-binding protein, resulting in an increase in intracellular cAMP levels in rat anterior pituitary corticotrophs *in vitro* (Aguilera *et al.*, 1983). Further evidence that CRF receptors are linked to a guanine nucleotide-binding protein was demonstrated by Grigoriadis & De Souza (1989) and Webster & De Souza (1988) when they found that the specific binding of [^{125}I]-CRF to rat pituitary , brain and spleen homogenates was increased in the presence of divalent ions such as Mg^{2+}, whereas the addition of increasing concentrations of guanosine-5' triphosphate (GTP) inhibited the binding. *In vitro* studies indicate that Mg^{2+} helps to stabilise the high affinity form of the receptor-effector complex, while GTP promotes the dissociation of the receptor protein from the guanine nucleotide-binding protein.

Initial studies into the biochemical characteristics of the CRF receptor revealed that the molecular mass of the pituitary and the brain receptor differed in a number of species i.e. rodents, pig and human, (Grigoriadis & De Souza, 1988). In the anterior pituitary and intermediate lobe the apparent molecular mass was 75 kDa, while in the rat brain the CRF-binding activity resided in a protein with an apparent molecular mass of 58 kDa (Grigoriadis & De Souza, 1989). This receptor heterogeneity between the brain and the anterior pituitary was subsequently discovered to be the result of differential posttranslational glycosylation of the native protein, since deglycosylation generates polypeptide with comparable molecular weights, 40 to 45 kDa.

Recently a cDNA encoding the pituitary CRF receptor was cloned from a human Cushing's adenoma (Chen *et al.*, 1993b), designated $CRFR_1$. Subsequently, a cDNA of the rat homologue (Perrin *et al.*, 1993) was cloned and this shares 97%

CRF Receptor Subtype	Distribution	Pharmacological profile
$CRFR_1$	CNS - cerebral & cerebellar cortexes, amygdala & hippocampus Pituitary	CRF_{41}> Urocortin >> Sauvagine >>Urotensin
$CRFR_{2\alpha}$	CNS - lateral septum, ventromedial hypothalamic & medial amygdaloid nuclei	Urocortin >>Sauvagine >> Urotensin > CRF_{41}
$CRFR_{2\beta}$	Periphery - duodenum, skeletal muscle, epididymis & heart CNS - associated with blood vessels	Urocortin >> Sauvagine >> Urotensin > CRF_{41}

Table 1.1: The distribution and pharmacological profiles of the CRF receptor subtypes: $CRFR_1$; $CRFR_{2\alpha}$; and $CRFR_{2\beta}$ in the adult rat..

identity at the amino acid level. An *in situ* hybridisation study using a riboprobe generated from the rat $CRFR_1$ cDNA demonstrated that CRF receptor mRNA expression was present in several regions of the adult rat brain (Wong *et al.*, 1994). It is a member of a distinct family of seven transmembrane domain receptors (Chang et al., 1993), which include calcitonin, vasoactive intestinal peptide and parathyroid hormone receptors (Serve & Goldring, 1993).

De Souza and colleagues recently cloned and characterised a novel CRF receptor, designated CRF-R_2, from a rat hypothalamus cDNA library (Lovenberg *et al.*, 1995b). This receptor was demonstrated to exist as two splice variants, termed CRF-$R_{2\alpha}$ and CRF-$R_{2\beta}$ which differ in their amino-terminal sequence. Using RNase protection assay and *in situ* hybridisation techniques the expression of the mRNA for CRF-$R_{2\alpha}$ was demonstrated to be almost exclusively in the brain, particularly in the hypothalamus, lateral septum and olfactory bulb, whereas the mRNA for CRF-$R_{2\beta}$ was present in both the brain, where it is thought to be associated with blood vessels, and in peripheral tissues, such as the heart and skeletal muscle (Lovenberg *et al.*, 1995a). In the mouse only the homologue of $CRF_{2\beta}$ has been cloned and is expressed almost exclusively in the heart and skeletal muscle (Kishimoto *et al.*, 1995; Perrin *et al.*, 1995). The genomic structure and corresponding cDNA sequence of the human $CRF_{2\alpha}$ receptor were cloned and characterised (Liaw *et al.*, 1996) from human genomic cDNA libraries. Its pharmacological profile was comparable to that of the rat $CRF_{2\alpha}$ receptor. However, the genomic and cDNA sequences encoding a human homologue of the rat $CRF_{2\beta}$ remain to be elucidated.

In addition to the CRF receptor subtypes differing in their anatomical distribution, they also exhibit unique pharmacological profiles. CRF is more potent at stimulating cAMP production in isolated anterior pituitary cells via $CRFR_1$ than either sauvagine which was isolated from the skin of the South American frog *Phyllomedusa sauvagei* (Montecucchi *et al.*, 1980) and possesses more than 50% homology with oCRF, or urotensin I, isolated from the urohypophysis of two species of fish, *Catostomus cyprimus* and *Catostomus catostomus.* (Lederis *et al.*, 1982). However, both sauvagine and urotensin are more potent than CRF at $CRFR_{2\alpha}$ and $CRFR_{2\beta}$. This led to the hypothesis that in mammals another CRF-like compound

may be the natural ligand for these receptors. This was confirmed by the recent isolation from the rat brain of a peptide called urocortin, which possesses homology with sauvagine and urotensin and has a high affinity for both $CRFR_{2\alpha}$ and $CRFR_{2\beta}$ but not $CRFR_1$ (Vaughan *et al.*, 1995). Recently the human urocortin has been cloned and characterised (Donaldson *et al.*, 1996).

1.3.4 Regulation of CRF receptors

The interaction between CRF and its specific receptor is a crucial event in the activation of the HPA axis since it initiates the synthesis and release of ACTH and other POMC gene products. Therefore, several *in vivo* and *in vitro* studies have looked at the regulation of the pituitary CRF receptor, which has been identified by *in situ* hybridisation studies to be the $CRFR_1$ subtype (Potter *et al.*, 1994).

It is well known that chronic exposure of a tissue to the actions of its trophic hormone can result in a reduced sensitivity of the organ to the same hormone. This agonist-induced desensitisation has been demonstrated in several endocrine systems and represents an adaptive response to prolonged hormone exposure (Catt *et al*; 1979).

Studies using radioreceptor binding techniques, functional assays and hybridisation techniques have demonstrated that CRF possesses the ability to regulate its own receptors in both the pituitary and brain. Pre-treatment of rat primary anterior pituitary cell cultures with CRF (10^{-7} M) *in vitro* causes a rapid reduction in its ability to subsequently stimulate cAMP synthesis and ACTH release (Reisine & Hoffman, 1983). Another study found that there was a 36% decrease in CRF-binding sites when dissociated fetal brain cell cultures were exposed to oCRF (10^{-6} M) for three days. *In vivo*, high levels of CRF administered by the intracisternal (i.c) or intracerebroventricular (i.c.v) route significantly reduced the number of CRF receptors measured by a competitive binding assay. Using northern blot and RNase protection analyses, CRF *in vitro*, dose- and time- dependently decreased $CRFR_1$ mRNA levels in cultured rat anterior pituitary cells (Sakai *et al.*, 1996; Pozzoli *et al.*, 1996). In the same study, the cells were treated with increasing doses of AVP. This caused a similar reduction in CRF receptor expression although the decrease was

longer lasting. Since CRF and AVP are coreleased into the hypophyseal portal blood and the latter potentiates the ACTH-releasing activity of the former, some investigators have looked at the effect of AVP on $CRFR_1$. Aguilera's group demonstrated *in vivo* that the loss of CRF pituitary receptors observed after exogenous CRF administration was enhanced by a simultaneous infusion of AVP (Tizabi & Aguilera, 1992), however only at doses that elevated plasma AVP to levels in the range of those in the hypophysial portal circulation (Hauger & Aguilera, 1993). The same group also demonstrated that the potentiated downregulation by AVP occurred after repeated stress and not after 60 day water-restriction indicating that chronic endogenous activation of the parvocellular but not the magnocellular vasopressinergic system is responsible for this enhancement (Hauger & Aguilera, 1993).

Adrenalectomy (adx) caused a decrease in CRF_1 receptor binding (Wynn *et al.*, 1984; Hauger *et al.*, 1987) and mRNA expression in the anterior pituitary (Sakai *et al.*, 1996; Makino *et al.*, 1995a), presumably caused by the increased release of CRF and AVP into the portal circulation following adx (Holmes *et al.*, 1986b). The adx-induced changes in CRF receptors can be completely reversed by glucocorticoid replacement with dexamethasone (De Souza *et al.*, 1985b). However, numerous studies have investigated the effect of glucocorticoid treatment on CRF receptors expression in intact animals and found an opposite effect to that in adrenalectomised animals. Chronic administration of low doses of corticosterone (Hauger *et al.* 1987) or high doses of the synthetic glucocorticoid, dexamethasone (Wynn *et al.*, 1985) in intact rats causes a dose-dependent decrease in the binding of [^{125}I]oCRF to the anterior pituitary. This led to the proposal that since low doses of naturally occurring glucocorticoids downregulate anterior pituitary CRF receptors this may be of physiological importance in the inhibitory action of glucocorticoids on ACTH release and may reflect a general inhibitory effect of glucocorticoids on the corticotroph. Thus, as well as causing a rapid decrease in POMC gene transcription (Eberwine & Roberts, 1984) glucocorticoids may also decrease the synthesis of intrinsic proteins such as the CRF receptors. This was subsequently confirmed by Pozzoli *et al* (1996), who demonstrated that treatment of cultured anterior pituitary cells with either

dexamethasone or corticosterone time- and dose-dependently decreased receptor mRNA expression. Similarly, *in vivo* Makino *et al* (1995a) found that following chronic administration of corticosterone the $CRHR_1$ receptor mRNA expression was reduced in the anterior pituitary. However, a receptor binding study by Hauger *et al* (1987) found that although chronic corticosterone administration downregulated CRF receptor binding in the anterior pituitary it did not affect receptor expression in brain regions including, the cortex, amygdala and hippocampus. This result led to the hypothesis that central CRF receptors are differentially regulated by corticosterone. However, recently Makino *et al* (1995a) demonstrated that administration of exogenous corticosterone via either daily injections or from slow-releasing sc pellets resulted in a decrease in $CRFR_1$ mRNA in the PVN and lateral and basolateral amygdaloid nuclei but not in the central nucleus of the amygdala or BNST. This difference between studies may reflect the use of sensitive hybridisation techniques in the latter study allowing the detection of small changes in mRNA levels which may or may not be translated to the protein level.

Both acute and chronic stress cause a downregulation in CRF receptor mRNA in the anterior pituitary (Luo *et al.*, 1994) while upregulating receptor expression exclusively in the PVN (Makino *et al.*, 1995a). In addition, i.c.v infusion of CRF results in an upregulation of $CRFR_1$ subtype specific to the PVN (Mansi *et al.*, 1996), providing evidence for a positive feedback effect of CRF on its own receptor in the PVN (Silverman *et al.*, 1989). However, other studies using whole hypothalamic organ culture showed that activation of CRF receptors reduces the release of CRF (Calogero *et al.*, 1988). Therefore, the former observation may be due to CRF diffusing away from the injection site and acting on other hypothalamic or extrahypothalamic sites that send projections to the PVN.

Several mechanisms have been proposed for the CRF receptor downregulation. These include internalisation of the cell surface receptors (Catt *et al.*, 1979). This is supported by the observation that labelled CRF is internalised into corticotrophs (Leroux & Pelletier, 1984). Interestingly, it has been hypothesised that the potentiation of the CRF-induced downregulation by AVP may be mediated via activation of the PKC pathway causing phosphorylation of the receptor and

promoting internalisation (Holmes *et al.*, 1987). Alternatively, diminished receptor binding capacity due to uncoupling of the receptor from the adenylate cyclase has also been postulated (Reisine & Hoffman, 1979). However, numerous studies examining the modulation of CRF receptor gene expression by various agents including CRF, AVP and glucocorticoids indicate that at least one of the important mechanism of downregulation is reduced receptor mRNA expression (Makino *et al.*, 1995a; Pozzoli *et al.*, 1996). Interestingly, a recent study demonstrated that the cAMP and PKC pathways are involved in the modulation of the $CRFR_1$ mRNA levels in the anterior pituitary possibly acting at both the transcriptional and posttranscriptional level (Pozzoli *et al.*, 1996).

1.3.5 Corticotropin-releasing hormone binding protein

The presence of a CRF binding protein (CRFBP) in the plasma of pregnant women was confirmed by chromatographic studies by Linton *et al* (1988) where it acts to bind plasma CRF, which circulates at high concentrations particularly during the third trimester of pregnancy (Goland *et al.*, 1986; Sasaki *et al.*, 1984), and reversibly neutralises its biological activity. It is a glycoprotein produced by the liver (Suda *et al.*, 1989a) and has a molecular mass of approximately 40 kDa (Linton *et al.*, 1988). Although it is also present, but at much lower levels, in the plasma of nonpregnant females and males it appears to be absent from the plasma of rats and sheep (Linton & Lowry, 1986; Linton *et al.*, 1988). However, both rat and human cDNAs encoding the CRFBP have been isolated (Potter *et al.*, 1991). Potter *et al* (1992) carried out an extensive immunohistochemical and hybridisation study examining the central distribution of the CRFBP and found the highest expression occurred in the cerebral cortex with lower levels in certain subcortical limbic structures including, the amygdaloid complex and the BNST, sensory relays associated with the auditory, olfactory, vestibular and trigeminal systems and several raphe nuclei in the brainstem. Dual immunostaining studies revealed limited colocalisation of CRF and CRFBP. Interestingly, in several cell groups of the forebrain CRFBP-immunoreactive terminal fields were in close association with several CRF-immunoreactive cell bodies indicating that at select central loci CRFBP

may be released from terminals to act to modify local synaptic, autocrine or paracrine actions of CRF. However, in the PVN only a few scattered cells expressed CRFBP-immunoreactivity or mRNA. This suggests that at the level of the PVN the CRFBP does not influence the activity of CRF. In contrast, CRFBP is highly expressed in the anterior pituitary corticotrophs, suggesting that at this site the binding protein, via an undefined mechanism, may modulate the actions of CRF on ACTH release.

1.3.6 Arginine Vasopressin

Arginine vasopressin (AVP) is a nonapeptide hormone which was isolated and characterised more than 40 years ago (Du Vigneaud *et al.*, 1953). Its classic role is in fluid and electrolyte balance, therefore changes in plasma osmolality, blood pressure or blood volume are the main stimuli for AVP secretion into the peripheral circulation (for review see Robertson, 1992). It is synthesised as a larger prohormone in magnocellular neurones in the posterior region of the PVN and in the supraoptic nucleus (SON) of the hypothalamus (Brownstein *et al.*, 1980). It is carried by axonal transport via the internal layer of the median eminence to nerve terminals in the posterior pituitary, where it is stored for later release into the peripheral circulation.

The vasopressin gene is approximately 2kb in size and consists of three exons and two introns, encoding a signal peptide, vasopressin, neurophysin II and a carboxy-terminal glycopeptide (Schmale *et al.*, 1983). Following transcription, splicing and translation the prehormone, preprovasopressin, is cleaved by signal peptidases, making provasopressin, which contains vasopressin, neurophysin II and the carboxy-terminal glycopeptide. The prohormone is processed into neurosecretory granules through the Golgi apparatus, during which it undergoes postranslational modifications such as processing, amidation and glycosylation to yield the mature peptide secretory products (Richter, 1988).

In addition to its presence in magnocellular neurones, AVP is present in the parvocellular neurones of the medial region of the PVN which project to the external lamina of the median eminence (ME). At the ME AVP is stored and released into the HPB system, where it regulates ACTH secretion from the anterior pituitary (for review see Antoni, 1986). Many laboratories have reported that following adx in rats,

between 70% and 90% of parvocellular CRF immunoreactive neurones exhibit immunoreactivity for vasopressin (Kiss *et al.*, 1984b; Sawchenko *et al.*, 1984a) and contain the mRNA for the vasopressin prohormone (Wolfson *et al.*, 1985). At the level of the median eminence 95% of CRF-containing axons and nerve terminals contain AVP following adx (Whitnall *et al.*, 1987b).

In intact rats, early studies indicated that AVP was expressed in less than 2% of the parvocellular cell bodies in the PVN (Kiss *et al.*, 1984b; Sawchenko *et al.*, 1984a). However, a more recent study using antibodies against AVP precursor-derived peptides (pro-AVP peptides) demonstrated that more than half of the parvocellular CRF-containing cell bodies stained positively for pro-AVP peptides. In addition the AVP-containing and AVP-deficient CRF neurones were distributed differently within the parvocellular PVN, with the former concentrated in the dorsal medial region (Whitnall & Gainer, 1988). Previous studies by the same investigators found a similar result in the ME where approximately half of the CRF-containing parvocellular axons and nerve terminals of the median eminence contained AVP precursor-derived peptides packaged in the same neurosecretory granule as CRF (Whitnall *et al.*, 1985). Therefore, the parvocellular CRF neurones can be divided into two major subpopulations, based on the expression of pro-AVP peptides and a differential distribution with the PVN.

Early work indicated that AVP and the yet undiscovered hypothalamic CRF synergised to stimulate ACTH secretion from the anterior pituitary (Gillies & Lowry, 1979; Yates *et al.*, 1971). Following the isolation of CRF several laboratories confirmed the potentiating effect of AVP on CRF-induced ACTH release *in vitro* (Beny & Baertschi, 1982; Gillies *et al.*, 1982; Guguere & Labrie, 1982) and *in vivo*, in rats pretreated with chlorpromazine, pentobarbital and morphine, which strongly inhibits endogenous CRF secretion but preserves the anterior pituitary responsiveness to exogenous substances (Arimura *et al.*, 1967).

As in the case of other peptide hormones, AVP exerts its regulatory effects through interactions with specific plasma membrane receptors of which there are two main receptor types, V_1 (VR_1) and V_2 (VR_2) (Jard *et al.*, 1987). The VR_2 are localised on the renal tubular epithelial cells (Jard, 1988) where they mediate the antidiuretic

effect and are coupled to adenylate cyclase. The VR_1 can be subdivided: VR_{1a}, the classical pressor receptors are located on vascular smooth muscle cells and are responsible for the vasopressor effects of AVP and on hepatocytes (Jard *et al.*, 1987; Michell *et al.*, 1979). They mediate increases in the cytosolic Ca^{2+} concentrations via activation of the phosphoinositide pathway (Jard, 1988). VR_{1b} which are expressed primarily on the anterior pituitary (Antoni, 1984; Baertschi & Friedli, 1985) can be distinguished from the VR_{1a} subtype by different ligand specificities (Antoni, 1984; Jard *et* al., 1986). The VR_{1b} is coupled to the calcium-calmodulin-kinase C pathway of signal transduction. This is supported by data showing that AVP enhances the breakdown of inositol phosphates in primary pituitary cell cultures (Raymond *et al.*, 1985) and a study using individual cells reported increased cytosolic free calcium and ACTH release following challenges with AVP (Leong, 1988).

One mechanism for the potentiation of the ACTH releasing actions of CRF by AVP may involve enhancing CRF-induced cAMP formation (Guguere & Labrie, 1982) by possibly inhibiting cyclic nucleotide phosphodiesterases. AVP does not appear to influence either the activation of adenylate cyclase by CRF (Gaillard et al., 1984) or its binding to CRF receptors (Holmes *et al.*, 1984).

The response of the parvocellular neurones expressing AVP mRNA to acute stress appears to vary from no response or a small increase (Darlington *et al.*, 1992; Harbuz *et al.*, 1994; Lightman & Young, 1988; Makino *et al.*, 1995b) to a more significant increase (Bartanusz *et al.*, 1994; Herman, 1995), indicating that this may reflect differences in the stress paradigm used. Other studies measuring changes in AVP primary transcripts or heteronuclear RNA (hnRNA) in the parvocellular PVN in response to stress consistently found an increase (Herman & Sherman, 1993; Ma *et al.*, 1997)

This stress-specificity is also seen in the release of AVP and CRF into the hypophysial portal blood. Romero *et al* (1993) found that in colchicine-treated rats insulin-induced hypoglycaemia but neither novelty nor restraint stress caused the release of AVP into the hypophysial portal blood. These stress-specific results are proposed to reflect the different populations of AVP-containing neurones in the parvocellular PVN and the fact that they are likely to be regulated by different inputs.

The role of AVP from magnocellular neurones in the regulation of ACTH release is controversial. In a stress paradigm such as haemorrhage or water deprivation, there are increased circulating AVP levels (Aguilera *et al.*, 1993; Robertson *et al.*, 1992) which may gain access to VR_{1b} on pituitary corticotrophs (Donhanics *et al.* 1991). However, the majority of previous studies suggest that the concentration of AVP in HPB is not altered following the removal of the posterior pituitary (Gibbs, 1985; Horn *et al.*, 1985). Hence, it was inferred that AVP released by magnocellular neurones, which have axon terminals in the posterior pituitary, does not reach the anterior pituitary in significant amounts. However, an *in vitro* study (Holmes *et al*, 1986a) indicated that preterminal magnocellular axons in passage through the ME may secrete AVP and *in vivo* PVN lesions did not significantly decrease the amount of AVP released into the HPB (Antoni *et al.*, 1990). In addition, exocytosis of neurosecretory granules along vasopressinergic axons in the internal lamina of the ME has been reported using the tannic acid fixation method (Buma & Niewenhuys, 1988). Although the latter result may reflect sprouting of SON axons within the ME.

AVP expression in the parvocellular PVN is negatively regulated by glucocorticoids (Davis *et al.*, 1986, Sawchenko, 1987a). A recent report using AVP luciferase reporter gene constructs transfected into the human choriocarcinoma cell line JEG-3 showed that dexamethasone suppressed gene activation but independently of the putative glucocorticoid response element (GRE) on the AVP promoter region (Iwasaki *et al.*, 1997).

1.3.7 Oxytocin

Oxytocin (OT) is also a nonapeptide hormone differing from the primary structure of AVP by two amino acid residues (a substitution of an isoleucine for a phenylalanine and a leucine for an arginine). The elucidated nucleotide sequence of the rat OT gene (Ivell & Richter, 1984) shows considerable homology both in organisation and sequence with the AVP gene. Like AVP, OT is synthesised as an inactive prohormone in the magnocellular neurones of the PVN, SON and suprachiasmatic nucleus (SCN) of the hypothalamus, axonally transported via the

internal lamina of the ME to the nerve terminals in the posterior pituitary where it is released upon appropriate stimulation into the peripheral circulation.

Only one study demonstrated the presence of oxytocinergic nerve terminals in the external lamina of the ME (Vandesande *et al.*, 1977) using an affinity purified antibody. They also reported that following lesioning of the PVN the OT-reactive nerve terminals in the external lamina of the ME disappeared. The cell bodies giving rise to these fibres could not be identified: magnocellular cell groups do not appear to project to the external lamina of the ME, and the parvocellular oxytocin neurones in the PVN appear to project to the spinal cord (Sawchenko & Swanson, 1985). Therefore, the only well-defined oxytocinergic cell groups that send axons to the ME are the magnocellular neurones of the PVN and SON.

In addition to its well-established role in parturition and lactation (Jenkins & Nussey, 1991), OT appears be involved in the regulation of stress-induced ACTH secretion from the anterior pituitary (Gibbs, 1986; Lang *et al.*, 1983). In the rat, OT can amplify the ACTH response to CRF, both *in vitro* (Antoni *et al.*, 1983a; Gibbs *et al.*, 1984a) and *in vivo* (Rivier & Vale, 1985). High affinity receptors for OT, which are clearly distinct from the V_{1b}, have been identified on rat anterior pituitary tissue (Antoni, 1986b) and appear to be coupled to mechanisms increasing free cytosolic Ca^{2+} concentrations by releasing it from intracellular stores which requires activation of phospolipase C and production of inositol 1,4,5-triphosphate (Berridge & Irvine, 1989). Several studies have shown that OT is released from the ME into the portal blood system (Antoni *et al.*, 1990; Tannahill *et al.*, 1991). In addition, the concentration of OT in the HPB is significantly higher than in the periphery (Gibbs, 1984b) as is the case with both CRF (Gibbs & Vale, 1982) and AVP (Koeing *et al.*, 1986). In a study in PVN-lesioned rats the release of OT into the HPB was reduced by 40% suggesting that a substantial amount of OT in the portal blood, as with AVP, is likely to be derived from preterminal release from magnocellular neurones (Antoni *et al.*, 1990). All this evidence reinforces the notion that OT functions as a hypophysiotropic hormone.

The release of OT from the posterior pituitary also appears to be an important component of the stress response. Rats exposed to acute stresses, including

immobilisation, ether, novel environmental, insulin-induced hypoglycaemia and swim stress as well as osmotic stressors, including intraperitoneal injection (i.p) of hypertonic saline all exhibit increased OT release into the circulation (Gibbs, 1984c; Lang *et al.*, 1983; Onaka & Yagi, 1993). A recent study examined the relative importance of the SON and PVN for OT release during stress (Jezova *et al.*, 1993). The plasma OT concentrations in rats with either incomplete anterolateral cuts (ALC) (leaving the fibres from the SON, but not the PVN, intact) or complete ALC cuts did not increase following 30 min of immobilisation stress. This indicates that at least in relation to immobilisation stress, the PVN is essential for stress-induced OT secretion and without the PVN the SON cannot preserve OT secretion during stress. Interestingly, CRF has been well documented as having a stimulatory action on OT release (Bruhn *et al.*, 1986) thus, during stress CRF may excite the OT-containing cells. However a report by Higuchi *et al* (1990) found that following CRF immunoneutralisation there was only a small reduction in OT release during immobilisation at 60-120 min after the onset of the stress, while the initial high levels of OT remained unchanged. This indicates that CRF may not have a prominent role in the stimulation of OT from the posterior pituitary during stress.

1.4 Pituitary Gland

The pituitary gland is a compound endocrine gland located in a depression in the floor of the cranial cavity and connected to the hypothalamus by a short stalk called the *infundibulum*. It can be divided into a glandular portion, the *adenohypophysis*, and a neural portion, the *neurohypophysis*. The adenohyphysis can be further subdivided into a *pars distalis*, which comprises the bulk of the adenohypophysis, a *pars tuberalis* and a *pars intermedia*. The neurohypophysis is divided into a *pars nervosa* and the infundibulum. The pars distalis and pars tuberalis are considered to form the anterior pituitary and the pars nervosa and pars intermedia the posterior pituitary gland.

Its blood supply is derived from the internal carotid artery. The posterior gland is supplied directly by branches of the two inferior hypophysial arteries. The branches of the superior hypophysial arteries supply the hypothalamus and the

infundibular stalk forming a capillaries plexus at this site. A system of veins arising from this plexus delivers blood to the anterior pituitary and is termed the hypophysial portal blood (HPB) system. It is via this HPB system that humoral substances, such as CRF and AVP from the hypothalamus are delivered to the anterior pituitary.

1.4.1 Anterior pituitary

The anterior pituitary secretes several trophic hormones, including growth hormone (GH), thyroid stimulating hormone (TSH), prolactin, follicle-stimulating hormone (FSH), luteinising hormone (LH) and adrenocorticotrophin (ACTH). It is composed of different cell types, reflecting the diversity of its functions. Prior to the discovery of the influence of the hypothalamus the anterior pituitary was seen as the 'master gland' coordinating numerous bodily functions, including growth, reproduction and metabolism. Corticotrophs are the cell type which secretes ACTH; however, they only account for approximately 5% of the cell population (Morrill *et al.*, 1993). ACTH is a 39 amino acid peptide which stimulates the synthesis and release of glucocorticoids from the adrenal cortex. It mediates it actions through G-protein coupled receptors resulting in the accumulation of intracellular cAMP which in turn increases the transcription of the P450 side-chain cleavage enzyme, the initial and rate-limiting step of adrenal steroidogenesis (for review see Miller, 1988).

1.4.2 Posterior pituitary

The posterior pituitary is derived from neural cells in a region from which the hypothalamus is also formed and consists of nerve fibres and terminals whose cell bodies lie in the hypothalamus, particularly in the PVN and supraoptic nucleus (SON) and modified astroglial cells, the pituicytes. Oxytocin (OT) and arginine vasopressin (AVP) are the two hormones secreted by the posterior gland.

1.5 Proopiomelanocortin

Proopiomelanocortin (POMC) is the precursor for a variety of biologically active neuroendocrine peptides. POMC mRNA is expressed in a variety of tissues,

including the hypothalamus especially the arcuate nucleus, amygdala, cerebral cortex (Civelli *et al.*, 1982), adrenal medulla (Jingami *et al.*, 1984), ovary and testes (Pinter *et al.*, 1984). However, its main site of expression is the pituitary gland in the corticotrophs of the anterior pituitary and the melanotrophs of the intermediate lobe (Civelli *et al.*, 1982). The POMC molecule is posttranslationally processed to different biologically active peptides in these two cells types: ACTH and β-lipotropin, which is further processed to the opioid peptide β-endorphin (Lazarus *et al.*, 1976) being the major products of the corticotrophs; and α-melanocyte stimulating hormone (α-MSH), corticotropin-like intermediate lobe peptide (CLIP) and acetyl β-endorphin of the melanotrophs in rats (Eipper & Mains, 1980).

Genomic DNA fragments containing the POMC encoding sequences have been isolated and sequenced from several mammalian species, including human (Takahashi *et al.*, 1983) and rat (Drouin *et al.*, 1985). The POMC gene is composed of three exons separated by two introns, with the third exon encoding all the biologically active peptides.

The first *in vivo* studies used cell-free translation followed by immunoprecipitation to indirectly quantify POMC mRNA levels in whole pituitaries. Nakanishi *et al* (1977) were the first to show that adx, a procedure known to stimulate ACTH release, caused a time-dependent increase in POMC levels which could be reversed by glucocorticoid replacement. Subsequent studies using hybridisation techniques demonstrated that the sensitivity of POMC mRNA levels to glucocorticoids was displayed almost exclusively in the anterior pituitary (Birnberg *et al.*, 1983; Bruhn *et al.*, 1984). Birnberg *et al* (1983) correlated the increase in plasma ACTH with increases in anterior pituitary POMC mRNA in the several hours to days following adx in the rat. They found that within eight hours of adx, plasma ACTH levels had increased and then dropped as the ACTH content of the anterior pituitary is depleted. This is followed by a gradual increase in the POMC mRNA content of the anterior pituitary followed by an increase in the plasma ACTH, reflecting the increase in synthesis and processing of the POMC peptides. Administration of dexamethasone transiently increased the pituitary content of POMC peptides most likely because it inhibits POMC peptide release, as seen in the

fall in plasma ACTH levels, more rapidly than it inhibits POMC peptide synthesis. Hence in this experiment, POMC mRNA correlated with secretion of POMC-derived peptides *in vivo*.

In situ hybridisation studies have found similar observations of adx and dexamethasone replacement on pituitary POMC mRNA expression. Fremeau *et al* (1986) found that 14 days after adx the level of silver grains detected over the corticotrophs was increased and that a short term dexamethasone treatment (100 mg, i.p.) for 30 min to 120 min had no effect on reducing silver grain density over the corticotrophs. Thus in agreement with the observation of Birnberg *et al* (1983) glucocorticoids require several hours to elicit their inhibitory effect on POMC mRNA levels.

Several *in vivo* studies have investigated the importance of the hypothalamic PVN input in the regulation of POMC mRNA levels. Bruhn *et al* (1984) lesioned the PVN, the major site of the CRF neurones projecting to the ME and showed a decrease in the levels of POMC mRNA in the anterior pituitary. This indicates that CRF and other hypothalamic factors may be responsible for the increase in POMC mRNA expression following adx. A study by Dallman *et al* (1985) demonstrated that *in vivo* the negative regulation of anterior pituitary POMC mRNA expression by corticosterone occurs in a region at or above the level of the hypothalamus. They found that following knife cuts that destroyed CRF and AVP input to the anterior pituitary the POMC mRNA levels were decreased, but more importantly replacing with increasing concentrations of corticosterone in these lesioned adx rats had no significant effect on the level of anterior pituitary POMC mRNA.

Stress, a potent stimulator of the secretion of POMC-derived peptides *in vivo*, has also been shown to stimulate POMC mRNA levels. Studies using either an intermittent electrical footshock stress paradigm showed an approximate 2-fold increase in POMC mRNA levels in the anterior pituitary after three days and this remained elevated at seven days (Hollt *et al.*, 1986), or three weeks of chronic arthritis pain caused an 80% increase in anterior pituitary POMC mRNA levels (Millan *et al.*, 1986b).

In general, studies into POMC gene expression using primary anterior pituitary cell cultures or ATt20 cells (murine tumour cell line) have tried to identify the mechanisms by which POMC gene expression is regulated at the cellular level.

The treatment of primary anterior pituitary cells or AtT20 cells with either corticosterone or dexamethasone causes a decrease in the level of POMC mRNA. Studies using AtT-20 cells found that dexamethasone was more potent than either cortisol or corticosterone in decreasing POMC expression and that its half-maximal dosage was 3 nM (Roberts *et al.*, 1979). Together these indicate that the glucocorticoid effects are probably mediated through glucocorticoid receptors. Similar effects were seen in primary cell cultures (Eberwine *et al.*, 1987). Thus, it appears that, at least in part, the effects of glucocorticoid treatment *in vivo* on anterior pituitary POMC mRNA expression are mediated by direct effects on the corticotroph. However, the magnitude of the glucocorticoid effect *in vitro* is only half that observed *in vivo*, suggesting that glucocorticoids are likely to be having effects at other levels of the HPA axis, such as on hypothalamic CRF gene expression (Jingami *et al*, 1985a).

CRF has been shown to have direct stimulatory effects on POMC gene expression in both AtT-20 and anterior pituitary cells in dispersed cell culture. CRF exposure for eight hours stimulated POMC mRNA levels approximately 3-fold in AtT20 cells, an effect mimicked by the addition of 8-bromo-cAMP, a cAMP analogue and phorbol esters, direct stimulators of protein kinase C (Affolter & Reisine, 1985). The effect on POMC gene expression of CRF and other agents which increase cAMP production, but not phorbol ester, could be blocked by fusion of AtT20 cells with liposomes containing a cAMP-dependent protein kinase inhibitor (Reisine *et al.*, 1985). This, demonstrates that the increase in POMC mRNA by CRF and cAMP analogues is due to activation of protein kinase A (cAMP-dependent kinase). Studies in primary cultures show that both CRF and forskolin increase the levels of POMC mRNA (Loeffler *et al.*, 1985), again supporting a common role for cAMP in regulating expression of the POMC gene. Meanwhile, activation of protein kinase C is able to increase POMC mRNA independently of activation of protein kinase A (Reisine *et al.*, 1985).

The release of POMC-derived peptides is calcium dependent so several groups have explored the calcium dependence of factors which stimulate POMC gene expression through cAMP-dependent processes. Loeffler *et al* (1986) found that basal levels of POMC mRNA levels in primary cultures were inhibited by prolonged incubation with the calcium channel antagonists D600, verapamil and nifedipine. In addition, the latter partially blocked the stimulatory effect of forskolin. Dave *et al* (1987) found that D600 did not effect basal POMC mRNA levels but significantly attenuated the CRF-induced increase. However, the mechanism by which cAMP- and Ca^{2+}-dependent processes interact to increase POMC gene expression is not known.

Several studies have examined whether the changes in POMC mRNA levels are due to increased mRNA synthesis i.e. increased rate of transcription or due to decreased degradation of mRNA. An *in situ* hybridisation study using an intronic POMC probe which allows the measurement of the nuclear primary transcript or heteronuclear RNA (hnRNA), demonstrated that the adx-induced increase of POMC mRNA levels in the anterior pituitary was the result of an increased transcription rate in individual cells and in the number of cells detectably transcribing the POMC gene (Fremeau *et al*., 1989). Several research groups have shown that glucocorticoids have a rapid inhibitory effect upon anterior pituitary POMC gene transcription (Eberwine & Roberts, 1984; Gagner & Drouin, 1985). The effects of dexamethasone are rapid, with a significant decrease occuring within 15 min unlike its effects on mRNA levels which require up to 48 h to maximally inhibit. However, corticosterone (500 μg i.p.) took 45 min to 120 min to maximally inhibit transcription and did not produce as large an inhibitory effect. The may reflect the presence of transcortin in the corticotrophs which avidly binds corticosterone, preventing its actions, but does not bind dexamethasone, thereby allowing the latter free access to GR while the *in vivo* effects of corticosterone are likely to occur at the hypothalamus and therefore will take longer to influence anterior pituitary POMC gene transcription. In addition, dexamethasone has a 5-10 times higher affinity for GR than either cortisol and corticosterone.

A number of studies in primary anterior pituitary cell cultures have clearly demonstrated that a physiological concentration of CRF rapidly stimulates POMC

gene transcription at times well before the observed CRF-induced increase in POMC mRNA levels (Gagner & Drouin, 1985; Eberwine *et al.*, 1987).

While both glucocorticoids and CRF have direct effects on corticotrophs to alter POMC gene transcription, the corticotroph *in vivo* is never exposed to only one of these hormones, rather it is under the influence of a combination of both in the intact animal. Gagner & Drouin (1985) measured POMC transcription in cultures simultaneously exposed to both CRF and dexamethasone and found transcriptional levels to be intermediate between control levels and those of CRF alone. Eberwine *et al* (1987) went on to evaluate transcription of the POMC gene in primary cultures in response to combinations of the two hormones under different orders of addition. Dexamethasone pretreatment for varying periods of time inhibited subsequent CRF-stimulated POMC gene transcription in a manner similar to the well established inhibitory effects of glucocorticoids on subsequent CRF stimulation of POMC-derived peptide release *in vitro* (Buckingham, 1979; Gillies & Lowry, 1978; Widmaier & Dallman, 1984). Pretreatment of the cultures with dexamethasone for 10 min had little inhibitory effect on subsequent CRF-stimulated POMC gene trancription, however, following 15 min of pretreatment the dexamethasone inhibitory effect appeared. On the other hand, CRF pretreatment for 60 min prevented the subsequent glucocorticoid inhibitory effect, while CRF pretreatment for 10 min allowed dexamethasone to be fully inhibitory on POMC gene transcription. Thus when POMC gene transcription is maximally activated, glucocorticoids cannot inhibit transcription. The molecular mechanism underlying this complex relationship is undefined; however, *in vitro* studies by Shipston & Antoni (1992) demonstrated that CRF inhibited the synthesis of calmodulin, a proposed effector of glucocorticoid feedback in AtT-20 cells, a murine corticotroph tumour cell line.

The potentiation of CRF-induced ACTH secretion by AVP *in vivo* (Arimura *et al.*, 1967; Lui *et al*, 1983) and *in vitro* (Giguere & Labrie, 1982) is well established. However, recent *in vitro* studies indicate that AVP does not potentiatate CRF-induced accumulation of either cytoplasmic POMC mRNA (Suda *et al.*, 1989b) or hnRNA (Levin *et al.*, 1989). More importantly, AVP by itself decreases POMC

hnRNA levels in primary anterior pituitary cell cultures. Thus *in vivo*, this novel inhibitory influence of AVP may allow a more refined regulation of POMC gene expression than would be accomplished by CRF alone.

1.6 Adrenal gland

The adrenal gland is located on the superior pole of each kidney and is composed of two distinct parts, a central *medulla* and an outer *cortex* which differ in their origin, function and structure. Each gland is enclosed in a thick, connective tissue capsule.

1.6.1 Adrenal cortex

The adrenal cortex is divided into three concentric layers: the *zona glomerulosa*, a thin, outer layer adjacent to the capsule; the *zona fasciculata*, a thick, middle layer; and the *zona reticularis*, a thin, inner layer adjacent to the medulla.

The parenchymal cells of the cortex secrete a variety of hormones collectively referred to as corticosteroids. The cells of the zona glomerulosa secrete a class of steroid hormones called mineralocorticoids. The most important one is aldosterone, which functions in the maintenence of normal electrolyte balance. The cells of the zona fasciculata, and to a lesser extent, those of the zona reticularis, secrete a class of steroid hormones called glucocorticoids. Cortisol, in humans and non-human primates, and corticosterone, in rats and mice, are the most important members of this class and influence carbohydrate, fat and protein metabolism. Finally the cells of the zona reticularis, and to a lesser extent those of the zona fasciculata, secrete small amounts of sex steroids, primarily androgens. Quantitatively the most important androgens secreted in humans are dehydroepiandrosterone sulphate, dehydroepiandrosterone and androstenedione although in rats and mice thier secretion is negligible.

1.6.2 Adrenal medulla

The chromaffin cells of the medulla secrete catecholamines, primarily adrenaline (epinerphine) and smaller amounts of noradrenaline (norepinerphine).

These substances produce effects similar to those induced by activation of the sympathetic division of the autonomic nervous system, including increased heart rate, respiration and blood pressure, reduced blood flow to viscera and skin, stimulation of the conversion of glycogen to glucose and decreased digestion. Their release into the peripheral blood circulation is initiated by preganglionic sympathetic fibres innervating the chromaffin cells.

1.7 Glucocorticoids

The major glucocorticoid in man, non-human primates and sheep is cortisol and in rodents it is corticosterone. Studies with isolated, perfused adrenals (Reichstein & Shoppee, 1943), analysis of urine from normal individuals (Conn *et al.*, 1951) or patients with Cushing's disease (Mason, 1950) and adrenal vein blood (Nelson *et al.*, 1951; Reich *et al.*, 1950) demonstrated that cortisol and corticosterone were the primary secretory products of the adrenal cortex. They are 21-carbon compounds synthesised from cholesterol and the hydroxy group at carbon-11 was discovered to be required for therapeutic efficacy (Kendall, 1941; Olson *et al.*, 1944).

Glucocorticoids are catabolic hormones which causes lipolysis, glycogenolysis and protein catabolism, which directly and indirectly, via gluconeogenesis, increase blood glucose levels (Munck, 1984). These actions assist the organism during stress by, at least in part, increasing the availability of energy substrates at the expense of existing energy stores and of anabolic processes. Glucocorticoids also suppress immunological responses (Munck, 1984), decreasing inflammation when mobility may be essential. However, prolonged exposue to elevated glucocorticoid levels can present a serious risk, leading to a suppression of anabolic processes, muscle atrophy, decreased sensitivity to insulin and a risk of steroid-induced diabetes, hypertension, hyperlipidemia, hypercholesterolemia, arterial disease, amenorrhoea, infertility and the impairment of growth and tissue repair as well as immunosuppression (Brindley & Rolland, 1989; Munck, 1984). Thus, once the stress is terminated it is in the organism's interest to 'turn-off' the HPA axis stress response, thus the circulating glucocorticoids feedback on the anterior pituitary and specific brain regions to inhibit

the further release of CRF and ACTH as discussed above (Dallman *et al.*, 1987; Keller-Wood & Dallman., 1984; Plotsky *et al.*, 1987b).

1.7.1 Corticosteroid Receptors

The majority of actions of glucocorticoids in the brain and periphery require trancription and/or translation, and therefore can take from minutes to hours to become manifest and are mediated by intracellular corticosteroid receptors. Two types have been isolated, and cloned: the type I or mineralocorticoid (MR) receptor (Arriza *et al.*, 1987; Patel *et al.*, 1990) and the type II or glucocorticoid (GR) receptor (Hollenberg *et al.*, 1985; Miesfeld *et al.*, 1984). They are members of the steroid/thyroid hormone receptor gene superfamily (Evans, 1988).

1.7.1.a Mode of action

In the absence of glucocorticoids, MR and GR are mainly cytoplasmic (Picard *et al.*, 1990; Robertson *et al.*, 1993) and exist in a multimeric complex with a number of accessory proteins (Hutchison *et al.*, 1994) which are proposed to maintain the receptors in a conformation that promotes ligand binding. Once steroid binding has occurred, the 'activated' receptor dissociates from the accessory proteins and migrates to the nucleus where it binds either as a homodimer (Glass, 1994) or as a heterodimer (Trapp *et al.*, 1994) to specific 'cis'-acting genomic DNA sequences, or glucocorticoid response elements (GREs) to either induce or repress the rate of transcription of target genes.

In the rat the DNA binding and ligand binding domains of both MR and GR show more than 90% homology to the human receptors (Arriza *et al.*, 1988; Miesfeld *et al.*, 1984; Patel *et al.*, 1990). Thus, both MR and GR have been highly conserved through evolution. Several studies have elucidated the primary structure of GR and MR which are comprised of 777 and 984 amino acid residues, respectively (Evans, 1988). Detailed studies have examined the structure of the GR. The amino-terminal or A/B domain is not conserved within the steroid/thyroid hormone receptor family and is proposed to be involved in mediating transcriptional regulation of target genes (Hollenberg *et al.*, 1987). It also contains the recognition sites for antisera. The DNA

binding or C domain, by contrast, is highly conserved (94% homology between GR and MR). It posssesses two 'zinc finger' motifs, which attach to GREs (Glass, 1994; Luisi *et al.*, 1991). Other residues in this domain are involved in the transactivation of target genes (Hollenberg *et al.*, 1987). The conserved D domain comprises approximately 42 residues and is proposed to be important for receptor translocation to the nucleus. The E domain which represents the carboxy-terminal is involved in binding the ligand. This region also binds accessory proteins, including HSP-90 (Chakraborti *et al.*, 1991), is involved in dimerisation (Fawell *et al.*, 1990) and has transactivation and nuclear translocation activities (Godowski *et al.*, 1988).

1.7.2 Corticosterone binding globulin (CBG)

Circulating CBG is present in most mammals, including man and the rat (Seal & Doe., 1965) with the liver being the major site of synthesis in the adult (Hammond et al., 1987; Smith & Hammond, 1989). Cloning and sequencing of the CBG cDNA has revealed that it belongs to the serine protease inhibitor family (Hammond *et al.*, 1987). It is the major plasma protein binding corticosteroids and during the course of the normal diurnal rhythm it has been estimated that approximately 90% of corticosterone is bound to CBG in the circulation (Hammond, 1990; Siiteri *et al.*, 1982). Therefore, variations in plasma CBG levels will affect the free concentration of glucocorticoids in the plasma and therefore influence the bioactive efficacy of the glucocorticoid signal. In addition, anterior pituitary cells contain an intracellular CBG, referred to as transcortin (de Kloet & McEwen, 1976; Koch *et al.*, 1976) which is proposed to be derived from plasma CBG. It exists in a membrane-bound form as well as in the cytosol (Koch *et al.*, 1978), where it functions much like plasma CBG in that it sequesters free corticosterone and decreases binding to GR in the anterior pituitary. Plasma levels of CBG are known to be influenced by several hormones, including glucocorticoids, thyroxine and ovarian hormones (D'Agostino & Henning, 1982; Feldman *et al.*, 1979). Adx increases while chronic corticosterone treatment decreases plasma CBG levels (Feldman *et al.*, 1979; Levin *et al.*, 1987). More recently it has been reported that acute stress can influences both plamsa and intracellular levels causing a significant decrease 24 h after the end of the stress

(Tannenbaum *et al.*, 1997). Plasma CBG levels and hepatic mRNA levels in female rats are higher than those found in male rats (Smith & Hammond, 1989) and estrogen treatment enhances CBG levels in both man and the rat (Blomback *et al.*, 1983; Gala & Westphal, 1965). In support of a stimulatory influence of estrogen on CBG levels, during pregnancy the levels increase (Seal & Doe, 1967). The influence of estrogen is at least in part mediated via the sex-specific pattern of growth hormone secretion (Jansson *et al.*, 1989).

1.8 Feedback regulation of the HPA axis

Glucocorticoids, corticosterone in rodents and cortisol in man, negatively regulate both the basal and stress-induced activity of the HPA axis in a closed loop manner. Under *in vivo* conditions at least three distinct time domains have been identified in which glucocorticoids exert a negative feedback signal on stimulated ACTH secretion: fast rate-sensitive; intermediate; and slow feedback (for review see Keller-Wood & Dallman, 1984).

The existence of fast, rate sensitive feedback was first postulated by Dallman & Yates (1969) and occurs within seconds to minutes of glucocorticoid exposure when the plasma levels of the hormone are rising. However the effects only last as long as the plasma levels are rising at a sufficient rate (Jones *et al.*, 1972). Several *in vitro* studies have demonstrated that glucocorticoids have rapid effects on both stimulated CRF and ACTH secretion (Buckingham & Hodges, 1977; Vale & Rivier, 1977; Vermes *et al.*, 1977). While the early *in vivo* studies were based on administration of exogenous glucocorticoid and measuring indirect indices of ACTH secretion (Dallman & Yates, 1969; Kaneko & Hiroshige, 1978; Yates *et al.*, 1972), a more recent study demonstrated physiological evidence for the rate-sensitive fast feedback inhibition of ACTH secretion (De Souza & Van Loon, 1989). They used a 2 min restraint stress paradigm to elevate corticosterone levels and then applied a second restraint during the period when the plasma corticosterone levels were significantly rising. They found that although the maximum plasma ACTH concentrations were not different, the rate of decline was significantly greater following the second restraint. This can be interpreted as indicating that rate-sensitive feedback appears to

act to terminate and limit the duration, but not the peak, of the ACTH response to subsequent stress. Although the mechanism of inhibition is not known, the rapidity of the effects indicate that protein synthesis is not involved. In support of this, pretreatment of perifused pituitaries with cycloheximide, a protein synthesis inhibitor, did not prevent rapid inhibition (Widmaier & Dallman, 1983). Thus, this rapid steroid effect is not mediated by association with MR or GR, the classical intracellular receptors. Accumulating evidence suggests that rapid corticosteroid effects may be mediated through specific membrane receptors. Work by Orchinik and colleagues using amphibian brain membrane preparations support the existence of membrane receptors (Orchinik *et al.*, 1991). They demonstrated using radiolabelled ligand binding studies that [^{3}H]corticosterone binding to neuronal membranes was specific, saturable, reversible and of high affinity (0.14 nM). Further support for the existence of a proteinaceous binding site was that specific binding was eliminated by both heat denaturation and protease treatment. In addition it displayed a distinct pharmacological profile differing from the intracellular corticoid receptors. It was highly specific for corticosterone and cortisol, but possessed a low affinity for both dexamethasone and aldosterone. *In vitro* receptor autoradiographic studies and subcellular fractionation studies indicated that the binding sites were located in the region of the synaptic terminals and appear to be linked to a G protein mediated second messenger system. The existence of corticosteroid membrane receptors in the mammalian brain has been postulated. Chen *et al.* (1993a) and Towle & Sze (1983) described low affinity corticosterone binding sites in the rat brain and proposed that these membrane receptors may be involved in rapidly responding to high levels of corticosterone that occur during stress. However, their existence still remains controversial.

Dallman & Yates (1969) also hypothesised the existence of a delayed feedback effect of corticosteroids on ACTH secretion which was dependent on the duration of exposure to elevated levels of corticosteroids. One report found that *in vitro* incubation of AtT-20 cells (a murine pituitary tumour cell line) with dexamethasone for 5-25 h caused a significant decrease in ACTH release, however, following 2-3 days of dexamethasone exposure, both the amount of ACTH released and the ACTH

cell content were significantly reduced (Watanabe *et al.*, 1973). This led to the hypothesis that the glucocorticoid delayed feedback exerts distinct effects over time. Subsequently, delayed feedback was divided into intermediate and slow feedback effects. Intermediate feedback requires corticosterone exposure for 2-10 h previously and affects ACTH secretion but not synthesis. However, the mechanism of intermediate feedback on ACTH secretion does require DNA-dependent RNA and protein synthesis since the suppressive effects of the steroid are prevented in the presence of either actinomycin D, a RNA polymerase inhibitor or cycloheximide (Arimura *et al.*, 1969; Philips & Tashjian, 1982). In addition, intermediate feedback decreases CRF synthesis (Buckingham, 1979; Sato *et al.*, 1975). Slow feedback by corticosteroids results from constant corticosterone exposure for 12 h or longer and inhibits both ACTH release and synthesis. *In vivo*, glucocorticoid injections reduces the pituitary POMC mRNA levels in adx rats (Nakanishi *et al.*,1977) and *in vitro*, dexamethasone, cortisol and corticosterone specifically inhibit mRNA coding for POMC without altering the synthesis of other proteins (Roberts *et al.*, 1979). See 1.5 Proopiomelanocortin.

Several experiments have studied the inhibition of basal ACTH release, and suggest that this release is less sensitive to corticosteroid feedback; and that the timecourse of steroid inhibition of basal secretion differs from the timecourse of inhibition of stimulated secretion. Studies using incubated or perfused rat pituitaries with concentrations of either dexamethasone or corticosterone which suppress the ACTH response to exogenous CRF or hypothalamic-stalk median eminence extract do not inhibit basal release (Buckingham & Hodges, 1977; Widmaier & Dallman, 1983). In studies designed to investigate the rapid inhibition of ACTH, no inhibition was observed (Widmaier & Dallman, 1983). However, inhibition of basal ACTH secretion does occur when monolayer cultures of pituitary cells are incubated with corticosterone or dexamethasone for longer than four days (Fleischer & Rawls, 1970) or with supraphysiological concentrations (Kraicer & Milligan, 1970). *In vivo*, inhibition of basal ACTH secretion by glucocorticoids has also been demonstrated. In normal human subjects cortisol infusions inhibited basal ACTH secretion (Reader *et al.*, 1982). This difference between the *in vitro* and *in vivo* data suggests a

difference in the mechanism of ACTH secretion. Basal ACTH secretion *in vitro* may represent autonomous pituitary secretion while *in vivo* secretion may be being stimulated by inputs from the brain.

Stress-induced ACTH release *in vivo* is inhibited by fast feedback within seconds of the rise of plasma corticosteroids. It is then followed by a 'silent' period during which no inhibition of adrenocortical responses to stress is observed, then 90 to 120 min after the rise in plasma corticosterone intermediate feedback is observed. In rats, however, inhibition of basal pituitary-adrenal activity has been demonstrated during the 'silent' period (Zimmerman & Critchlow, 1972). Inhibition of plasma ACTH concentrations occurred 20 min after the onset of corticosteroid infusions and continued over the next 90 min after the end of the 40 min infusion. In human subjects plasma ACTH concentrations are reduced within 45 min after the onset of a cortisol infusion and the plasma ACTH concentrations remained suppressed as long as the plasma cortisol levels were high (Reader *et al.*, 1982). Thus basal ACTH secretion in normal animals is inhibited by corticosteroids but the temporal pattern of inhibition is different from that of stimulus-induced secretion. The inhibition of basal ACTH release *in vivo* is likely to represent of inhibition of the CRF neurone and/or the afferent pathways mediating CRF stimulation under basal conditions.

1.9 Chronic Stress and Facilitation

The charateristics of the response of the HPA axis appears to depend on the type of stress used. Several studies have demonstrated that exposure of rats to either repeated (daily intermittent) or chronic (continuous) stress causes elevations in plasma corticosterone levels, increased adrenal gland weight and changes in peripheral corticosterone-sensitive tissues, such as decreased thymus gland weight (Hauger & Aguilera, 1992; Hauger *et al.*, 1988; Kiss & Aguilera, 1993). All these are indicative of chronic activation of the HPA axis. However, regardless of this chronically stressed animals appear to be fully responsive to new acutely applied stress (Hauger & Aguilera, 1992; Hauger *et al.*, 1988, 1990; Vernikos *et al.*, 1982). Studies into the sensitivity of the HPA axis to exogenous corticosterone have used adx animals replaced with varying doses of corticosterone. It was found that chronic

exposure to even the lowest dose of corticosterone decreased the amplitude of the ACTH response to stress in the morning and prevented a response to a subsequent stress (Akana *et al.*, 1992). Similarly, to determine the role of CRF and vasopressin in the enhanced pituitary responsiveness to a novel stress following chronic activation of the HPA axis, Tziba & Aguilera (1992) gave CRF or CRF in conjunction with AVP by subcutaneous infusion for 50 h, then exposed the rats to acute ether or restraint stress. Unexpectedly, it was discovered that the animals exposed to the chronic ACTH secretagogue treatment had a lower ACTH response than control rats. Thus, it was hypothesised that chronic administration of exogenous corticosterone or ACTH secretagogues inhibit further activity of the HPA axis but endogenous secretion following stress does not, therefore stress itself must facilitate subsequent responses of the system (Dallman, 1993).

Numerous studies have demonstrated that chronic stress increases (Kiss & Aguilera, 1993; Makino *et al.*, 1995b; Mamalaki *et al.*, 1992) CRF mRNA expression in the parvocellular PVN. However, other studies failed to find any upregulation (Harbuz *et al.*, 1992). This discrepancy between studies is likely to be a consequence of using different stressors. Interestingly there is evidence suggesting that during repeated or chronic stress there is a shift in the modulation of pituitary-adrenal activity from CRF to AVP (Hashimoto *et al.*, 1988; Scaccianoce *et al.*, 1991). Immunohistochemical studies have shown with chronic intermittent stress that there is a progressive increase in the AVP content of nerve endings in the external zone of the median eminence (De Goeij *et al.*, 1991) and that the enhanced immunoreactivity is due to an increase in the number of AVP-positive CRF nerve terminals (Bartanuz *et al.*, 1994, De Goeij *et al.*, 1991; Whitnall, 1989). In a recent study exposure to repeated immobilisation for 14 days resulted an increase in AVP mRNA content greater than that seen for CRF mRNA (Makino *et al.*, 1995b). Several studies suggest that the release of CRF and AVP into the portal circulation may be increased during chronic stress (Berkenbosch & Tilders, 1986; Whitnall, 1989). This is supported by the finding of Brown & Sawchenko (1997). It was shown that CRF neurones displayed a sustained immediate early gene response to insulin-

induced hypoglycaemia over 5 days of treatment, indicating chronic activation of these specific neurones.

It has previously been shown that chronic immobilisation decreases the number of CRFR, without affecting the Kd, in the anterior pituitary (Hauger *et al.*, 1988, 1990), although not during exposure to cold stress (Hauger & Aguilera, 1992). This may indicate enhanced release of CRF into portal blood or may represent an effect of the higher circulating glucocorticoid levels seen in animals that are chronically stressed. However, *in vitro,* anterior pituitaries have been shown to be more sensitive to CRF from chronically stressed animals (Young & Akil, 1985). Thus there appears to be a discrepancy between CRFR density and responsiveness of the pituitary corticotrophs. This indicates that a post-receptor event may be responsible for the change in pituitary responsiveness during chronic stress. A study to examine the effect of chronic stress on components of the second messenger system linked to the CRFR found that following 12 h of intermittent cold, swim stress the adenylate cyclase (AC) activity and mRNA expression was significantly increased in the chronically-stressed animals. This indicates that this enhanced AC activity may be important in maintaining the responsiveness of the HPA axis during chronic stress (Morrill *et al.*, 1993).

The potentiating effects of AVP on CRF-stimulated ACTH release is well known (Gillies & Lowry, 1979) and as mentioned above AVP expression appears to increase substantially during a chronic stress paradigm (De Goeij *et al.*, 1991). A recent report examined the relationship between AVP receptor levels in the anterior pituitary and AVP-stimulated ACTH release *in vitro* in rats subjected to chronic stress paradigms. It was found that following 14 days of either repeated i.p. hypertonic saline or immobilisation the binding of [^{3}H]AVP to anterior pituitary membrane-rich fractions was significantly increased and that AVP potentiated the CRF-induced ACTH secretion more from acutely dispersed anterior pituitary cells isolated from the chronically-stressed rats (Aguilera *et al.*, 1994).

As previously mentioned the circulating levels of corticosterone are substantially elevated in rats exposed to repeated or chronic stress. However, this continuous elevation is not seen in basal ACTH levels. Kiss & Aguilera (1993) found

that after 13 days of i.p. hypertonic saline injections, although the corticosterone levels were high the plasma ACTH levels were not signifiantly elevated. Similarly, Makino *et al* (1995b) found no elevation in ACTH following repeated immobilisation stress. It was proposed that during repeated stress less ACTH is required to transduce the centrally mediated call for glucocorticoid secretion from the adrenals that have become hyperplastic and hyperresponsive to ACTH. This phenomenon was demonstrated by Culman *et al* (1991) by giving systemic injections of CRF to repeatedly stressed rats, and demonstrating that the corticosterone but not the ACTH response was greater.

Alterations in glucocorticoid negative feedback have also been demonstrated to occur during chronic stress and may account, at least in part, for the persistence of the HPA axis resposiveness during chronic stress (Vernikos *et al.*, 1982; Young *et al.*, 1990). A recent study reported a decrease in GR mRNA expression in both the hippocampus and PVN in chronically stressed animals and proposed that this reduction in GR mRNA levels possibly indicates a reduced negative feedback signal and that this may be responsible for enhanced expression of AVP, since AVP mRNA levels appear to be more sensitive to glucocorticoids than CRF mRNA, in chronically stressed animals (Makino *et al.*, 1995b). However, it was noted that this decrease in GR mRNA levels may not necessarily reflect changes in receptor binding or function since previous studies had shown dissociation between GR mRNA levels and receptor binding (Bohn *et al.*, 1994; Chao *et al.*, 1989). Interestingly, previous reports found no changes in hippocampal or PVN GR mRNA after seven days of immobilisation stress (Mamakali *et al.*, 1992) or a reduction in GR mRNA only in CA1-2 or CA1 & CA3 in socially stressed rats (Chao *et al.*, 1993). Again the different duration or different type of stressor may account for these discrepancies.

Dallman and colleagues (Akana & Dallman, 1992) proposed that if stress produces facilitation of subsequent stress-induced ACTH secretion that is normally balanced by the corticosteroid signal secreted by the adrenals at the time of the initial stress, then this would be revealed under conditions in which the adrenals cannot secrete normal amounts of corticosterone. To obtain direct evidence that stress facilitates subsequent stress responses they carried out a series of experiments using

cyanoketone (CK), an inhibitor of 3β-hydroxysteroid dehydrogenase . CK treatment prevents the normal corticosterone response to an acute stress but maintains a corticosterone circadian rhythm of normal amplitude. Basal and stress-induced plasma ACTH and corticosterone were measured in rats stressed during the previous 12 h-period and in naive rats i.e not exposed to prior stress, at either the trough or peak of the circadian rhythm. It was found that CK-treated rats previously stressed at the peak of the circadian rhythm hypersecreted ACTH after a subsequent stress at the trough and ones that had been previously stressed in the trough had elevated basal peak ACTH levels, compared to either their naive controls or vehicle-treated rats. These results provide direct evidence that prior stress induces facilitation of subsequent activity in the HPA axis. They proposed that the site(s) of facilitation occurs in parallel and with access to the CRF neurones because neither corticosterone feedback nor facilitation induced by prior stress was observed on stress responses provoked at the time of the peak basal, or on basal levels at the time of the trough. One study indicating an influence of a neural input to the CRF neurones of the PVN during chronic stress demonstrated that local administration of tetrodotoxin, which blocks voltage-dependent Na^{2+} channels and thus nerve impulses, during chronic footshock stress prevented the increase in CRF and AVP mRNA (Sawchenko *et al.*, 1993).

Catecholaminergic inputs to the PVN were initially proposed to be the site of facilitation since during chronic stress the activity increased in several of the catecholaminergic cells groups in the medulla and pons, including the NTS and LC (Lachur *et al.*, 1991) and using microdialysis noradrenaline activity in the PVN is increased in repeatedly stressed rats (Pacak *et al.*,1992); chronic stress increases LC tyrosine hydroxylase, the rate limiting enzyme which converts tyrosine to dihydroxyphenylalanine, protein concentration and neuronal firing rate (Melia *et al.*, 1992). Interestingly, CRF fibres innervate the LC (Sawchenko 1987a) and during chronic stress CRF content in the LC increases (Chappell *et al.*, 1986). Repeated infusions of CRF antagonist near the LC blocked the induction of TH induced by repeated intermittent stress (Melia & Dunman, 1991). However, the LC only has a moderate input to the CRF PVN neurones (Plotsky *et al.*, 1989) and a study by

Murakami *et al* (1997) using an inhibitor of dopamineβ-hydroxylase, which prevents the conversion of dopamine to noradrenaline, clearly demonstrated that catecholamines did not play a major role in generating facilitation. Recently, Bhatnagar *et al* (1995) measured *fos* mRNA expression in rats previously exposed to chronic intermittent cold stress and found increased expression in several of the subnuclei of the amygdaloid complex. Consistent with this observation is the observation that CRF mRNA in the amygdala increases with increasing plasma levels of corticosterone (Makino *et al.*, 1994). Therefore, these results support the notion that the amygdala complex may be a potential site for the phenomenon of stress-induced facilitation.

1.10 The circadian rhythm of the HPA axis

Under normal conditions several mammals, including man and rodents, exhibit a circadian or diurnal rhythm in corticosterone, ACTH and CRF (Kwak *et al.*, 1992). The fluctuations in plasma glucocorticoids occur with the peak and nadir generally coinciding with the initiation and the termination of the animal's active period, respectively. Thus, in the rat, high levels of corticosterone are detected in the evening as they awaken while the levels are at their lowest in the morning as they enter their resting period.

Basal activity during the trough of the circadian rhythm is proposed to occur as a result of constitutive secretory activity of the pituitary and adrenal gland without hypothalamic input. On the other hand basal activity during the peak of the rhythm requires input from the hypothalamus driven by the suprachiasmatic nucleus (SCN), which appears to be the major pacemaker controlling the circadian HPA axis rhythm (Szafarczyk *et al.*, 1980). These propositions are supported by several findings. Firstly, following lesions of the basal hypothalamus (Kaneko *et al.*, 1980), the PVN (Dallman *et al.*, 1989b) or the SCN (Cascio *et al.*, 1987), the morning ACTH levels do not decrease, but the normal evening rise in ACTH and corticosterone is prevented. Secondly, passive immunisation of rats with CRF antisera did not alter trough ACTH and corticosterone levels but prevented the normal evening rise in both (Bagdy *et al.*, 1991). The circadian rhythm of ACTH and corticosterone follow each

other closely. The circadian drive appears to become activated during the early afternoon resulting in a rise in plasma ACTH which is closely followed by a comparable rise in corticosterone. Peak ACTH levels are attained close to the start of the active phase while corticosterone peaked about an hour or so into the dark phase (Kwak *et al.*, 1992). The SCN possesses both direct and indirect inputs to the PVN, at least in part via vasopressinergic neurones (Watts *et al.*, 1987) and bilateral lesions of the SCN prevent the daily increasing phase of ACTH secretion (Szafarczyk *et al.*, 1979). In adddition, neuromorphological and pharmacological data indicate that catecholaminergic projections from the brainstem appear to modulate the diurnal HPA rhythm, possibly via the CRF neurones since lesions of the ventral noradrenergic bundles prevent the normal daily rise in plasma ACTH levels (Szafarczyk *et al.*, 1985). Evidence also suggests that the mesencephalic serotoninergic system innervating the SCN may have a stimulatory influence since lesions prevented the normal ACTH rise (Szafarczyk *et al.*, 1979) while GABAergic interneurones may have an inhibitory influence on the circadian rhythm of the HPA axis (Ixart *et al.*, 1983).

A study by Kwak *et al* (1992) found that CRF mRNA expression in the PVN exhibited a diurnal rhythm. Increased CRF mRNA synthesis appears to occur in advance of ACTH and corticosterone secretion and persisits despite the increase in steroids in the afternoon. This increased trend in CRF mRNA during the light phase is consistent with previous reports on bioactive CRF concentrations in the hypothalamus and median eminence (Owens *et al.*, 1990; Szafarczyk *et al.*, 1980). In addition, Hiroshige and Sakakura (1971) have found a close temporal relationship between bioactive CRF activity in the median eminence and corticosterone in plasma, with corticosterone immediately following changes in CRF concentrations. Kwak *et al* (1992) also reported a sharp fall in CRF mRNA expression at the onset of darkness, thus CRF expression was inversely related to plasma corticosterone at the peak of the circadian rhythm. This may indicate that steroid inhibition decreases CRF mRNA levels as the effects of steroid feedback overcome the circadian drive to the CRF neurones. However, a subsequent study by Kwak *et al* (1993) found that adx did not prevent the decrease in CRF mRNA, thus, indicating that this effect occurs

via a steroid-independent mechanism. Work by Jacobson *et al* (1989) found that even in the absence of steroids (in adx rats) the plasma ACTH levels still exhibited a diurnal rhythm which reached a peak at 18.00 h and then declined thereafter, suggesting that the diurnal drive weakens after the onset of the dark phase. Thus, the diurnal oscillation of CRF mRNA levels may be regulated primarily by the presence or absence of circadian drive rather than by corticosterone feedback. Although resting concentrations of both ACTH and corticosterone exhibit a diurnal rhythm the amplitudes of these oscillations are different, with corticosterone increasing several fold greater than ACTH, resulting in a dissociation between plasma ACTH and corticosterone in the evening (Kaneko *et al.*, 1981). This observation led to the hypothesis that the diurnal changes in corticosterone are due to diurnal changes in the responsiveness of the adrenal to ACTH. It has subsequently been found that the diurnal variation in adrenal responsiveness for ACTH *per se* is not responsible for the diurnal variation in the plasma corticosterone levels. Rather, it appears that an increased sympathetic drive via the splanchnic nerve contributes to the corticosterone diurnal variation (Dijkstra *et al.*, 1996). It was found that splanchnic nerve transection significantly reduced resting evening corticosterone levels. This finding is supported by earlier work in dogs and calves which revealed that stimulation of the splanchnic nerve results in increased corticosterone output from the adrenal glands, but only when physiological levels of ACTH were present in the circulation (Edwards & Jones, 1987b; Engeland & Gann, 1989). Also transection of the splanchnic nerves reduced adrenal sensitivity to both endogenous and exogenous ACTH (Edwards & Jones, 1987a).

Studies in the rat have determined the mean daily basal plasma corticosterone levels to be approximately 5 μg/dl (Akana *et al.*, 1992) and it appears that the HPA axis actively defends this concentration. A study by Akana *et al* (1992) using intact rats implanted with pellets containing different concentrations of corticosterone (0%, 20%, 40% & 80% corticosterone) demonstrated that as the levels of corticosterone were passively elevated the rise in the morning corticosterone levels were counteracted by a decrease in the peak evening levels, thus allowing the maintenance of the mean daily circulating levels. Studies on the adequate replacement of

corticosterone in adx rats have revealed that morning basal ACTH levels are restored to normal with circulating free corticosterone levels of approximately 2nM, and that the IC_{50} for corticosterone on ACTH is 0.7 nM while during the evening peak, the IC_{50} increases to 3.9 nM. Several laboratories have investigated the roles of the high affinity MR (Kd = 0.5 nM) and the lower affinity GR (Kd = 2.5-5 nM) in the regulation of the basal and stress-induced activity of the HPA axis (Bradbury *et al.*, 1991; Dallman *et al.*, 1989a). Under basal morning conditions, it is proposed that ACTH secretion is regulated via occupancy of MR (Akana *et al.*, 1988). However, both receptor types appear to be involved in the negative control of HPA axis function at the evening peak and during stress (Bradbury *et al.*, 1994; Ratka *et al.*, 1989).

Thus, the control of the circadian rhythm of the HPA axis and the maintenence of a constant circulating mean daily level of corticosterone essential for normal functioning of corticosterone involves complex interactions between stimulatory drives and inhibitory feedbacks.

1.11 11β-Hydroxysteroid dehydrogenase

The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) plays an important role in corticosteroid physiology by regulating the access of glucocorticoids to both GR and MR (Table 1.1). To date two isoforms have been isolated and characterised: 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2).

11β-HSD1 is a microsomal enzyme (Koerner, 1969) which catalyses the interconversion of corticosterone to its inactive 11-keto metabolite, 11-dehydrocorticosterone in rodents and cortisol to cortisone in man. It utilises the pyrridine nucleotide, NADP as a cofactor. Its Kd for corticosterone and cortisol is in the micromolar range (Lakshmi & Monder, 1988) and it has a ubiquitous distribution.

More recently, a second isoform has been isolated, 11β-HSD2. It exclusively catalyses the dehydrogenase reaction i.e inactivation of corticosterone and cortisol. Its activity is dependent on the presence of the pyrridine nucleotide, NAD . It possess

	11β-HSD1	**11β-HSD2**
Apparent mol. wt.	34 kda	40 kda
Tissue distribution	Widespread part. liver & CNS	Kidney. colon & placenta
Co-substrate	NADP(H)	NAD(H)
Reaction direction in:		
- homogenates	bidirectional	dehydrogenase
- intact cells	primarly reductase	dehydrogenase
Km (nM):		
- cortisol	17000	50
- corticosterone	2000	10
- cortisone	200	>>
- 11-dehydrocorticosterone	250	>>

Table 1.2: Details of the two 11β-HSD isozymes: 11β-HSD1 & 11β-HSD2.

a much higher affinity for both corticosterone and cortisol, in the nanomolar range, than the type 1 (Brown *et al*, 1993) and it has a much more selective distribution.

1.11.1 11β-HSD1

In the 1950s sufficient quantanties of cortisol became available to allow studies into its metabolism. Burstein and colleagues (1953) found that oral administration of cortisol acetate to humans resulted in the secretion of 11-oxo C_{21} and C_{19} steroids in the urine and in the same year an enzyme responsible for catalysing the metabolism of cortisol to cortisone was discovered in rat liver (Amelung *et al.*, 1953). It was later termed "11β-hydroxy dehydrogenase" (Hubener *et al.*, 1956) but is now known as 11β-hydroxysteroid dehydrogenase (11β-HSD). In the early 1960s Osinski *et al* (1960) reported that 11β-HSD activity was dependent on the presence of the pyrridine nucleotide cofactors: NADP for 11β-dehydrogenase activity and NADPH for 11β-reductase activity. However, several studies clearly showed that the oxidation and reduction of the C_{11} of glucocorticoids was not uniformly distributed among tissues. Liver exhibited predominantly reductase activity while the kidneyshows a high dehydrogenase activity (Burton & Turnell, 1968). This led to the conclusion that the differences in glucocorticoid metabolism in tissues was due to either the presence of separate enzymes or tissue specific effects by a unique enzyme (Bush & Mahesh, 1959). The physiological role of 11β-HSD activity is to act as a crucial pre-receptor signalling pathway for corticosteroid receptors, maintaining *in vivo* the specificity of the kidney and colonic MR for aldosterone (Edwards *et al.*, 1988; Funder *et al.*, 1988) and regulating the access of corticosteroid to GR (Whorwood *et al.*, 1993).

It was originally proposed that 11β-dehydrogenase and 11β-reductase activities existed as two separate enzymes in at least the fetal lung (Abramovitz *et al.*, 1982) and the liver (Lakshmi & Monder, 1985). In the late 1980s Lakshmi & Monder (1988) successfully purified 11β-HSD from rat liver microsomes, and showed that the purified enzyme *in vitro* possessed exclusively 11β-dehydrogenase activity, thereby lending support to the 'two enzyme theory'. Subsequently, however, Agarwal *et al* (1989) screened a rat liver cDNA library, using a rabbit anti-11β-HSD

serum, and isolated a cDNA for 11β-HSD. When the cDNA was transfected into the chinese hamster ovary cell line it exhibited equal 11β-dehydrogenase and 11β-reductase activity, thereby demonstrating that both activities were encoded by a single protein. The 'liver-type' 11β-HSD or 11β-HSD1 is a 34 kD glycoprotein (Lakshmi & Monder, 1988) and belongs to the short chain alcohol dehydrogenase family (Krozowski, 1992). Numerous studies have shown that this enzyme has a wide tissue distribution both centrally and peripherally. *In vitro* studies, measuring predominantly dehydrogenase activity as it is known to be more stable then reductase activity *in vitro* (Lakshmi & Monder, 1985), have found the enzyme activity in peripheral tissues such as the liver, lung, kidney, ovaries and testis (Benediktsson *et al.*, 1992; Lakshmi & Monder, 1985; Nicholas & Lugg, 1982; Philips *et al.*, 1989). Immunohistochemical (Monder & Lakshmi, 1990) and *in situ* hybridisation studies (Low *et al.*, 1993; Tannin *et al.*, 1991) confirmed the presence of the enzyme protein in these tissues and as sites of enzyme synthesis, respectively. they were dependent on the addition of exogenous NADP to the reaction. This is in agreement with the finding that the brain maintains relatively low levels of pyrridine nucleotide cofactors compared to other tissues and may account for a subsequent finding of no detectable 11β-HSD activity in whole hippocampal extract (Edwards *et al.*, 1988; Funder *et al.*, 1988) since no exogenous NADP was used in the latter studies. More recent it has been clearly demonstrated that 11β-HSD activity and mRNA is present in a number of brain regions. Highest levels of activity and/or mRNA expression occurred in the hippocampus, hypothalamus, cerebral cortex, cerebellum and the anterior pituitary (Moisan *et al.*, 1990a, 1990b; Sakai *et al.*, 1992).

To account for the bidirectionality of this enzyme *in vitro* investigators have postulated that the direction of the enzyme *in vivo* may be controlled by the relative proportions of the oxidised to reduced pyrridine nucleotide i.e. NADP:NADPH ratio (Nicholas & Lugg, 1982; Torday *et al.*, 1976) within specific cellular compartments. Alternatively, local extremes in pH have also been postulated to influence the direction of the enzyme (Deckx & DeMoor, 1966). However, having such restrictions within the cell is rather an ineffective way to regulate the direction of one enzyme since these perturbations are also likely to influence other important enzymes

in the local enviroment. Although *in vitro* the enzyme will interconvert glucocorticoids, depending on which cofactor is present, there is mounting evidence using clonal cell lines and primary cell culture that *in vivo* 11β-HSD may act predominantly as a reductase (Jamieson *et al.*, 1995; Low *et al.*, 1994a; Rajan *et al.*, 1996). Thereby, enhancing rather than decreasing glucocorticoid access to GR.

1.11.2 11β-HSD2

The isolation and characterisation of the 11β-HSD1 enzyme was initially thought to answer the paradox of why *in vitro* recombinant MR showed an equally high affinity for both corticosterone, cortisol and aldosterone yet *in vivo* MR in the distal nephron selectively bound aldosterone despite a 1000-fold molar excess of circulating glucocorticoids (Krozowski & Funder, 1983). However, it became apparent that 11β-HSD1 was unlikely to be responsible for the aldosterone-selectivity of the receptors due to its low affinity for corticosterone, its relatively sparse expression in the kidney and its detection in the proximal tubules rather than being colocalised with the MR in the distal tubules and collecting ducts of the kidney (Rundle *et al.*, 1989).

Recently, a high affinity NAD-dependent enzyme was partially purified from human placenta (Brown *et al.* 1993) and cDNAs for this 11β-HSD2 have been cloned from human, sheep, rat & mouse (Albiston *et al.* 1994, Agarwal *et al.* 1994, Zhou *et al.* 1995, Cole, 1995). It has low nanomolar affinities for glucocorticoids, thus, making it more suitable for the physiological metabolism of corticosterone and cortisol. The purified protein has an apparent molecular weight of 40 kD. It is an exclusive dehydrogenase i.e. inactivates glucocorticoids, and is therefore proposed to be responsible for protecting the aldosterone-selective MR from occupation by glucocorticoids in tissues such as the kidney. This proposal is further supported by the finding of mutations and small deletions in the 11β-HSD2 gene in patients with the syndrome of apparent mineralocorticoid excess (Mune *et al.*, 1995; Wilson *et al.*, 1995) which results from illicit occupation of aldosterone-selective MR by cortisol. This reinforces the importance of the type 2 enzyme in protecting these receptors.

1.12 Pregnancy

During pregnancy the mother undergoes numerous physiological adaptations in order to support the developing fetus. Changes in water and electrolyte balance occur resulting in increased total body water and haemodynamic changes also occur, including increased cardiac output with the respiratory system also affected (Metcalfe *et al.*, 1988). In addition maternal metabolism is altered to provide the extra energy required to support the developing fetus. Since glucocorticoids are involved in the regulation of metabolism it is not too surprising to find that glucocorticoid regulation is also affected by pregnancy.

Studies in man and other animals indicate that plasma glucocorticoid concentrations progressively increase in pregnancy (Barlow *et al.*, 1974; Carr *et al.*, 1981), consistent with their role as mobilisers of energy stores (Widmaier, 1992). As far back as the mid-1960s, urine excretion of cortisol was shown to be elevated in pregnant women (Espiner, 1966) and later studies found a parallel increase in maternal plasma (Nolten *et al.*, 1980, Nolten & Rueckert, 1981). In the rat, Ogle & Kitay (1977) reported a fall in corticosterone levels early in pregnancy followed by an increase after midgestation to prepregnancy levels. A subsequent more detailed study in the pregnant rat clearly demonstrated a fall in the daily mean levels of corticosterone in early pregnancy which is followed by a progressive increase from midpregnancy to term (Atkinson & Waddell, 1995). This elevated glucocorticoid concentration in blood may simply reflect an increased secretion from the adrenal cortex or a reduced metabolic clearance rate (MCR). The MCR of glucocorticoids may decrease during pregnancy since the levels of CBG have been shown to rise (Rosenthal *et al.*, 1969). However, a recent a study by Waddell & Atkinson (1994) clearly demonstrated that the MCR in the rat was maintained at pre-pregnancy levels due to the additional transuterine extraction. In addition, the level of free, biologically-active cortisol has been shown to progressively increase with pregnancy (Demey-Ponsart *et al.*, 1982; Nolten & Rueckert, 1981). However, despite these elevated levels of cortisol and corticosterone in the blood both pregnant women and animals exhibit a normal circadian rhythm (Atkinson & Waddell, 1995; Nolten *et al.*,

1980) and are responsive to physical stress, including surgical procedures (Namba *et al.*, 1980; Neumann *et al.*, 1998).

To account for these elevated plasma glucocorticoid levels several workers suggested that progesterone may play a role. It was proposed that the high circulating levels of progesterone occurring during pregnancy might competively inhibit the binding of cortisol to GR, thereby preventing the feedback signal on ACTH release (Rosenthal et al., 1969). However, work on hypophysectomised pregnant non-human primates and rats found that both the adrenal weight and the plasma glucocorticoid levels were partially maintained as long as a viable placenta was left *in situ* (Knobil & Briggs, 1955, Hodgen *et al.*, 1975). These results strongly indicate that the placenta must release a substance possessing ACTH-like bioactivity.

Subsequently, the placenta has been found to synthesize a whole host of hormones normally of hypothalamic or pituitary origin including CRF and POMC-derived peptides such as ACTH and β-endorphin (Petraglia, 1991; Sirinathsinghji & Heavens, 1989).

1.12.1 Placental CRF

Extracts of human placenta were first shown to contain immunoreactive and bioactive CRF by Shibasaki *et al.* (1982). In humans, the placental concentration of immunoreactive CRF increases from week ten of gestation. Immunocytochemical studies have identified the site of CRF synthesis as the syncytiotrophoblast although staining also occurred in the amnion, chorion and decidua (Riley *et al.*, 1991). The gene expressing preproCRF is present from week seven of gestation but remains low until after midpregnancy and CRF mRNA is present with the transcript being of similar size to its hypothalamic counterpart (Frim *et al.*, 1988). Placental CRF mRNA concentration increases over twenty-fold during the third trimester which parallels the increase in the concentrations of CRF in the maternal plasma.

The placental CRF secretion is influenced by several different factors. In contrast to the effects of glucocorticoid on hypothalamic CRF synthesis and release (Kovacs & Mezey, 1987), treating a mixed population of term placental cells with dexamethasone or cortisol enhances both preproCRF mRNA levels and the release of

immunoreactive CRF (I-CRF) (Robinson *et al.*,1988). Prostaglandins ($PGF_{2\alpha}$ and PGE_2) also stimulate CRF secretion from cultured, term placental cells (Petraglia *et al.*, 1987). Other substances, including noradrenaline, interleukin-1β, oxytocin and AVP have all been shown to increase I-CRF release from cultured placental cells (Petraglia *et al.*, 1989), but whether these play important roles *in vivo* remains unclear.

During human pregnancy the concentration of I-CRF in the peripheral blood gradually increases from midpregnancy, progressing to a dramatic rise as term approaches (Goland *et al.*, 1986; Sasaki *et al.*, 1987). In contrast, neither the rat nor sheep exhibit high circulating levels during pregnancy and little evidence exists to suggest that CRF is synthesised in the placenta of either of these species.

In humans the placenta also secretes CRF into the fetal compartment, although at a slower rate than into the maternal compartment (Goland *et al.*, 1986; Sasaki *et al.*, 1987). There is also a positive umbilical venous-arterial difference in I-CRF concentrations at term (Goland *et al.*, 1986; Sasaki *et al.*, 1987), indicating placental secretion of I-CRF into the fetus.

It is not known to what extent placental CRF modulates the secretion of ACTH and other POMC-derived peptides from the maternal anterior pituitary. It has been proposed to contribute to the elevated cortisol plasma levels since a correlation between urinary free cortisol and maternal plasma CRF was observed during pregnancy (Goland & Warren, 1989). However, although plasma ACTH levels increase progressively with advancing pregnancy in humans (Carr *et al.*, 1981) this follows an initial fall so maximal levels during late pregnancy remain well within the normal nonpregnant range, which is in marked contrast to the high levels of circulating CRF found in late pregnancy. This has led several investigators to postulate that *in vivo* there are constraints on the biological activity of circulating CRF on the maternal anterior pituitary (for review see Thomson & Smith, 1989). It may simply reflect the rising levels of glucocorticoids interfering with the stimulatory effect of placental CRF on the anterior pituitary corticotrophs. Alternatively, the kinetics of the CRF exposure may alter the sensitivity of the corticotrophs. *In vivo*, the hypothalamus release CRF into the HPB system in a

pulsitile manner (Ixart *et al.*, 1987). In contrast, the anterior pituitary corticotrophs of pregnant women are exposed to a sustained high concentration of CRF, and this may serve to desensitize the anterior pituitary to CRF, via CRF receptor downregulation (Reisine & Hoffman, 1983). This is supported *in vivo* by the demonstration that pregnant women are insensitive to intravenous (i.v.) injections of CRF (Schulte *et al.*, 1988). Alternatively, the presence of the CRF binding protein (CRFBP) in human plasma in greatly increased amounts in pregnancy, but not in rodents or sheep, (Linton *et al.*, 1988) has been proposed to hinder the bioactivity of placental CRF. However, up to 38% of circulating CRF remains unbound and may thus affect pituitary function (Saminen-Lappalainen & Laatikainen, 1990). In addition, Suda *et al* (1988) found that the percentage of CRF bound CRF-BP decreases rapidly as term approaches, and Linton *et al* (1993) have found that the plasma concentration of the CRFBP declines in the last two weeks of pregnancy The placenta also secretes CRF into the fetal compartment, the levels of which increase towards term (Goland *et al.*, 1986). In summary, concentrations of I-CRF drastically increase in both maternal and fetal plasma during the third trimester of human pregnancy, paralleling increased placental expression of preproCRF gene and the higher levels of I-CRF in placental tissue. The rat does not produce placental CRF, so changes in the rat HPA axis in pregnancy cannot be related to this extrahypothalamic source of CRF.

1.12.2 Proopiomelanocortin in the placenta

A placental source of ACTH in humans had been postulated for a long time (Jailer & Knowlton, 1950). However, it was not until the mid-1970s that ACTH immunoreactivity (I-ACTH) and bioactivity (B-ACTH) in the human placenta were identified (Rees *et al.*, 1975). Subsequently β-endorphin immunoreactivity was also detected in placental extract (Nakai *et al.*, 1978; Odagiri *et al.*, 1979), strongly suggesting that POMC was synthesized in the placenta. This was confirmed by Krieger and her associates using the 'pulse chase method', where they 'pulsed' cultured trophoblastic cells with radiolabelled amino acids, then 'chased' the incorporation of radioactivity into the newly synthesised ACTH and β-endorphin.

Their results indicated that the placenta is not merely a storage site for POMC peptides but actively synthesises them (for review see Kreiger, 1982).

Immunohistochemical studies have localised I-ACTH within the placenta to the syncytiotrophoblast (Al Timimi & Fox, 1986). Interestingly, it is expressed in the same site as CRF, possibly indicating that paracrine interactions occur within the placenta. In both human and rats placental POMC expression appears to be constitutive as mRNA levels are maintained throughout pregnancy (Chen *et al.*, 1986); however, placental POMC mRNA is smaller than the pituitary POMC mRNA by approximately 200 bases (Chen *et al.*, 1986). This has led to the suggestion that its translatable product(s) may not be secreted into the maternal circulation and that POMC expression may be important to intracellular functioning rather than having a classic endocrine function (Clark *et al.*, 1990). In addition the post-translational processing of the POMC molecule also differs between the placenta and the pituitary. Size chromatography studies indicated that the ratio of α-MSH to ACTH was higher in the placenta (Krieger, 1982). Consistent with ACTH not being released from the placenta studies in humans have found that although plasma I-ACTH levels increase slightly during pregnancy they remain within the normal range. In addition, in the rat plasma ACTH levels are very low during pregnancy and no longer exhibit a diurnal rhythm (Atkinson & Waddell, 1995).

However, *in vitro* human placental tissue has been shown to be capable of secreting I-ACTH and treatment of cultured term placental cells or perifused term placental fragments (Petraglia *et al.*, 1987) with CRF stimulates the release of I-ACTH. It must be kept in mind that I-ACTH measurements may not reflect true ACTH levels as antibodies raised against ACTH can cross-react with other POMC-derived peptides, including β-endorphin. However, a recent study found that B-ACTH is released from placental tissue collected at both early and late gestation (Waddell & Burton, 1993). Interestingly, in the case of the sheep, the placenta appears to be capable of secreting POMC fragments into the maternal circulation in response to stress (Falconer *et al.*, 1988). Recent studies have shown that although the majority of B-ACTH released from perifused placental fragments is attributable to I-ACTH, other placental products also possess ACTH-like bioactivity (Waddell & Burton, 1993). In addition

several placental products, including prolactin, growth hormone and α-MSH appear to be able to stimulate the fetal adrenal cortex (Pepe & Albrecht, 1985; Rudman *et al.*, 1980). Thus, ACTH along with other POMC-derived peptides may *in vivo* be released from the placenta during human pregnancy but it appear unlikely that ACTH is released from the rat placenta. It may be the case that other POMC-derived peptides such as β-endorphin are more important during rat pregnancy.

1.13 Aims of the thesis

The first aim of this thesis was to determine whether during pregnancy the neuroendocrine response of the HPA axis to an acute stress was attenuated. Previous work had shown that the lactating rat was hyporesponsive to stress and that the CRF gene expression in the PVN was significantly reduced on day 21 of pregnancy compared to virgin rats. Taken together these indicate that the reproductive state may influence the activity of the HPA axis.

The second aim of this thesis was to investigate possible mechanisms, including decreased forward drive and enhanced sensitivity to glucocorticoid negative feedback, which may underlie this attenuation.

We examined the gene expression of the two major ACTH secretagogues, CRF and AVP, in the parvocellular neurones of the hypothalamic PVN under non-stress conditions. Evidence suggests that the sensitivity of the anterior pituitary corticotrophs to exogenous CRF is reduced during pregnancy, so we examined CRF binding site density in the anterior pituitary. In order to see whether this is reflected at the the second messenger level we measured the accumulation of cAMP following exposure to CRF. See Chapter 4 for discussion of studies.

We carried out a number of studies to determine whether the HPA axis of the pregnant rat was more sensitive to glucocorticoid negative feedback than the nonpregnant rat. We examined the gene expression of the two intracellular corticosteroid receptors, MR and GR, through which glucocorticoids mediate their effects, in the PVN and hippocampus, two well characterised glucocorticoid feedback sites.

The microsomal enzyme 11β-HSD1 catalyses the interconversion of glucocorticoids to their inactive keto-metabolites thereby regulating glucocorticoids access to MR and GR. It has a wide central distribution and is expressed in several of the classic glucocorticoid feedback sites including, the hippocampus, PVN and anterior pituitary. We measured the *in vitro* enzyme activity in homogenates of these tissues to see whether a change in enzyme activity occurred during pregnancy. Then, *in vivo* we examined the impact on the HPA axis stress responsiveness of inhibiting central 11β-HSD by the potent inhibitor, glycyrrhetinic acid.

Finally, in order to test the overall sensitivity of the HPA axis of the late pregnant rat we pharmacologically adrenalectomised rats using metyrapone, 11β-hydroxylase inhibitor and aminoglutethimide, 20α-hydroxylase inhibitor and then administered exogenous corticosterone and measured the fall in plasma ACTH concentrations. See Chapter 5 for discussion of studies.

The central endogenous opioid peptide system is widspread and is known to influence a variety of neuroendocrine neurones, including the hypothalamic magnocellular oxytocin neurones during pregnancy. We examined whether a similar enhanced opioid tone was occurring at the level of the hypophysiotropic neurones of the PVN of the pregnant rat by administering the opioid antagonist naloxone and measuring their stress response. See Chapter 6 for discussion of studies.

A close relationship exists between the HPA axis and the hypothalamic-pituitary-ovarian axis with each known to be able to influence the other. In fact, it is widely accepted that the ovarian steroids are responsible for the sexual dimorphism of the activity of the HPA axis: higher activity in females. Since the attenuated stress responsiveness occurs during pregnancy when the circulating levels of these steroids are high it is quite conceivable that they may play a role. To determine this we implanted virgin rats (ovaries intact) with s.c. estradiol and progesterone capsules and measured their stress response. In addition, we examined whether the dramatic fall in plasma progesterone levels in the last few days of pregnancy, due to degeneration of the corpus luteum, influenced the HPA axis. This involved removing the progesteone capsules two day prior to the stress experiment. See Chapter 7 for discussion of studies.

There is evidence demonstrating that during pregnancy in both humans and animals, such as the rat, the basal activity of the HPA axis is altered. It has previously been shown in human pregnancy that following administration of exogenous CRF less ACTH is secreted by the anterior pituitary and the axis appears less sensitive to dexamethasone suppression (Owens *et al.*, 1987; Shulte *et al*, 1990). Unlike the human the rat does not appear to have high circulating levels of CRF during pregnancy. Thus, this lack of contaminating placental CRF has allowed us to investigate changes which may be occurring centrally as well as at the anterior pituitary which may play a role in the attenuation of the stress responsiveness.

CHAPTER TWO

Materials & Methods

2.1 *In Vitro* Assays

2.1.1 11β-HSD 1 Enzyme Conversion Assays

2.1.1.a Tissue collection:

Virgin, day 16 and day 21 pregnant female Sprague Dawley rats were decapitated and the brain and anterior pituitary rapidly removed. The cerebellum was dissected and a small section of the outer cortex collected. The remaining brain was mounted onto a vibroslice (Camden Instruments) and 400 μm coronal sections were cut until an area containing the paraventricular nucleus (PVN) was reached (Fig 2.1). Then a 600 μm section was taken and placed in a petri dish on ice and the PVN was micropunched out of the section using a stainless steel needle with a flat tip (ID 1 mm). The remaining brain section was later stained histologically to verify the location of the PVN. Finally, a 300 μm section containing the anterior hippocampus was collected. All tissues were kept on ice until assayed.

2.1.1.b 11β-dehydrogenase Activity

11β-dehydrogenase activity was measured by a method modified from Moisan *et al.* (1990). Tissues were homogenised in a 1 ml homogeniser (BDH) in varying volumes of ice-cold assay buffer C, pH 7.7 (300 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5 (Sigma), 10% glycerol (BDH): 50 μl for PVN micropunches; 300 μl for anterior pituitary; 200 μl for hippocampus; and 500 μl for cerebellum. The protein concentration of each sample was determined colorimetrically (Bio-Rad protein assay kit), and then duplicate samples were incubated with 200 μM NADP

Fig 2.1: A photograph of a representative brain slice showing the micropunched PVN (**A**). The brain was cut using a vibroslice and an area around the PVN was removed using a stainless steel needle (ID 1 mm). **3V** - 3rd ventricle, **OC** - optic chiasm & **AH** - anterior hippocampus.

and 12 nM [1,2,6,7 ^{3}H]corticosterone (specific activity 81 Ci/mmol, Amersham International) at 37 °C.

In preliminary studies the optimal assay conditions were established. These were found to be a 60 min incubation with a protein concentration of 100 μg/ml for cerebellum, 200 μg/ml for hippocampus, 500 μg/ml for anterior pituitary and a concentration of 1000 μg/ml for the PVN micropunches (Figure 2.2). Each PVN micropunch determination was made on tissue pooled from two animals. To assess the backgound level of steroid conversion, blank samples (no protein) were run in parallel with each assay.

At the end of the incubation 1 ml of ethyl acetate was added and the samples centrifuged at 13,000 rpm for 5 min to extract the steroids. The upper aqueous layer of the samples was transferred into fresh eppendorf tubes and dried at room temperature in a fumehood overnight.

The steroids were then reconstituted in 100 μl of ethanol containing both unlabelled corticosterone and 11-dehydrocorticosterone (2.5 mg/ml). A 10 μl aliquot of each sample was pipetted onto a silica gel-coated aluminium thin layer chromatography plate (TLC, Merck Ltd) until a total volume of 40 μl was applied. Each TLC plate was divided into lanes allowing the loading of eight samples per plate. The plates were then run in sealed tanks containing a mixture of chloroform:95% ethanol (92:8) to separate corticosterone (Sigma) and 11-dehydrocorticosterone (Sigma). The steroid bands were visualised under ultra-violet light and scraped into eppendorf tubes containing 1 ml of Cocktail T scintillant (Amersham International).

The radioactivity of each steroid fraction was quantified in a β-counter and the data expressed as the percentage conversion of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone.

2.1.1.c 11β-reductase Activity

11β-reductase activity was determined by a method modified from Brown *et al.* (1993). The anterior pituitary and hippocampus were homogenised in Buffer C, pH

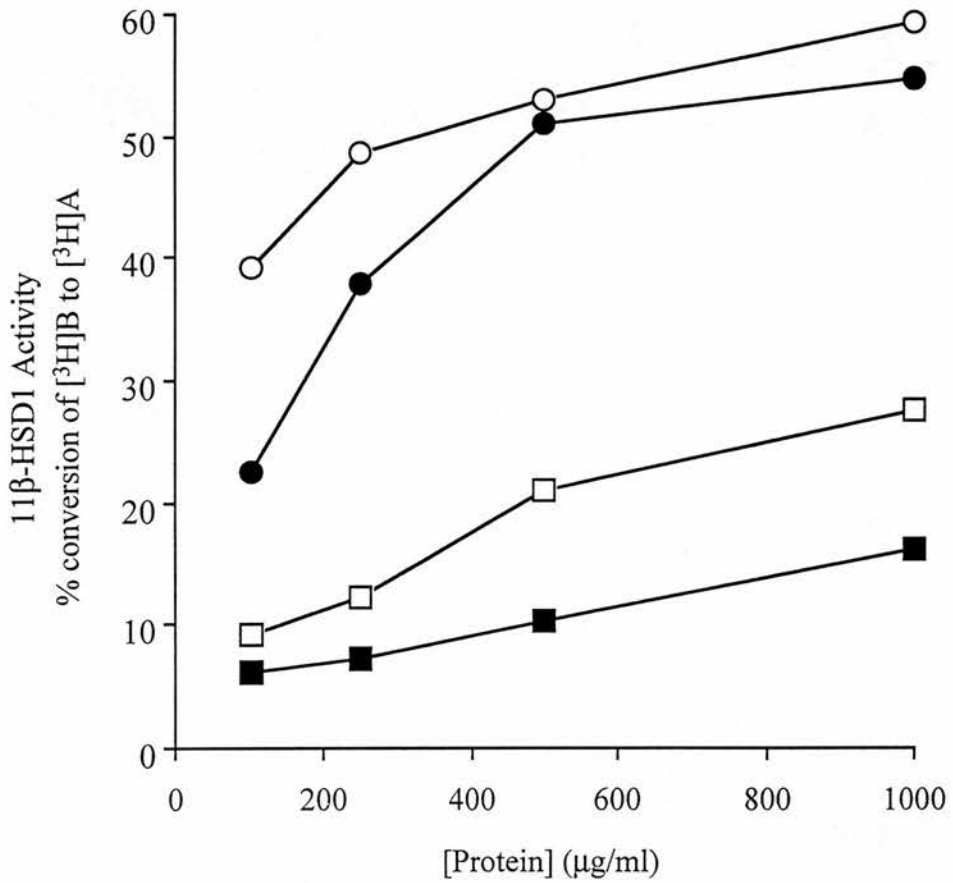


Fig 2.2: [Protein] vs. Incubation Time Curve. Increasing protein concentrations of (○) cerebellum, (●) hippocampus, (□) anterior pituitary & (■) hypothalamus homogenates were incubated with [^{3}H]corticosterone and 200 mM NADP for 60 min at 37 ^{o}C. Data expressed as the % conversion of [^{3}H]corticosterone (B) to [^{3}H]11-dehydrocorticosterone (A).

5-6 (300 mM NaCl, 1 mM EDTA 0.1 M potassium acetate, Sigma), and each homogenate incubated in duplicate in the presence of 200 μM NADPH and [^{3}H]11-dehydrocorticosterone for 10 min at 37 °C.

[^{3}H]11-dehydrocorticosterone was prepared from [^{3}H]corticosterone by a variation of the method of Lakshmi and Monder (1988). Human placenta as a pure source of 11β-dehydrogenase activity (Brown *et al.* 1993) and therefore producing the least contamination with corticosterone was used. Placental extract was incubated with 1 mM NAD in buffer C (pH 7.7) containing 12 nM [1,2,6,7 ^{3}H]corticosterone for a minimum of 2 h at 37 °C. The steroids were then extracted into an equal volume of ethyl acetate, centrifuged and the upper layer dried down under air. The sample was then resuspended in a volume of ethanol giving a specific activity equivalent to that of the [^{3}H]corticosterone used in the 11β-dehydrogenase assays, and stored at – 20 °C until required. To check the purity of the [^{3}H]11-dehydrocorticosterone, a sample of the steroids was dried down and resuspended in 600 μl of 65:35 methanol:HPLC H_2O and run through a Waters HPLC system. Purity of the sample was found to be greater than 98 %.

The steroids were extracted, separated and quantified by the same methods employed for the measurements of 11β-dehydrogenase activity. Data were expressed as the % conversion of [^{3}H]11-dehydrocorticosterone to [^{3}H]corticosterone.

In order to be able to compare data between assays we included liver microsomes, prepared by the method of Chirino *et al.* (1991), as internal standards in both the dehydrogenase and reductase assays. The liver was removed from an adult male Wistar rat and washed in ice-cold Buffer A, pH 7.4 (10 mM Tris, 1.5 mM EDTA, 2 mM dithiothreitol, 100 mM sodium molybdate, 80% glycerol). It was then cut into small pieces, weighed, transferred into a volume of fresh ice-cold Buffer A (3 x w/v) and homogenised. The homogenate was then centrifuged at 20,000 g for 20 min to sediment the larger pieces of tissue and the resulting supernatant centrifuged at 105,000 g for 60 min. The supernatant was discarded and the pellet resuspended in Buffer A and recentrifuged at 105,000 g for another 60 min. Again the supernantant was discarded and the pellet resuspended in a small volume of Buffer A. Protein

concentration was determined colorimetrically and the microsomes diluted down with Buffer A to give a protein concentration of 250 μg/ml, aliquoted and stored at – 80 °C until use.

The intra- and inter-assay coefficients of variation were 5% and 8 % and 9% and 10% for the dehydrogenase and reductase activity assays, respectively.

2.1.2 ACTH Two-site Immunoradiometric Assay

The plasma ACTH concentrations were measured using a commercially available kit (Euro-diagnostica B.V., The Netherlands), which employs two site-directed polyclonal antibodies, developed by Hodgkingson *et al.* (1984). This allowed the detection of the intact ACTH molecule without cross-reacting with related fragments.

Unextracted plasma and reconstituted human ACTH standards in plasma (5-1250 pg/ml) were incubated overnight at room temperature with a mix of [^{125}I]sheep anti-ACTH IgG and rabbit anti-ACTH IgG antibodies. The two antibodies reacted non-competitively as the sheep IgG was directed against the amino terminal region of the full-length human ACTH molecule while the rabbit IgG was specific to the carboxy-terminal.

Separation of the bound from the free ACTH was achieved by the addition of a precipitating reagent containing a sheep anti-rabbit IgG antibody followed by a short 30 min incubation at room temperature. This reagent caused the precipitation of the ^{125}I sheep antibody bound to the intact ACTH rabbit antibody complex. Finally, a wash solution was added and the samples centrifuged at 2,000 rpm for 20 min at 4 ^{o}C. The resulting supernatant was aspirated to waste and the wash, centrifugation and aspiration steps repeated. Standards and sample were quantified in a γ-counter, with the radioactivity in the precipitated bound fraction being directly proportional to the concentration of ACTH in the sample. The data were calculated from the standard curve (Figure 2.3) using the Microsoft MultiCalc programme and expressed in pg/ml.

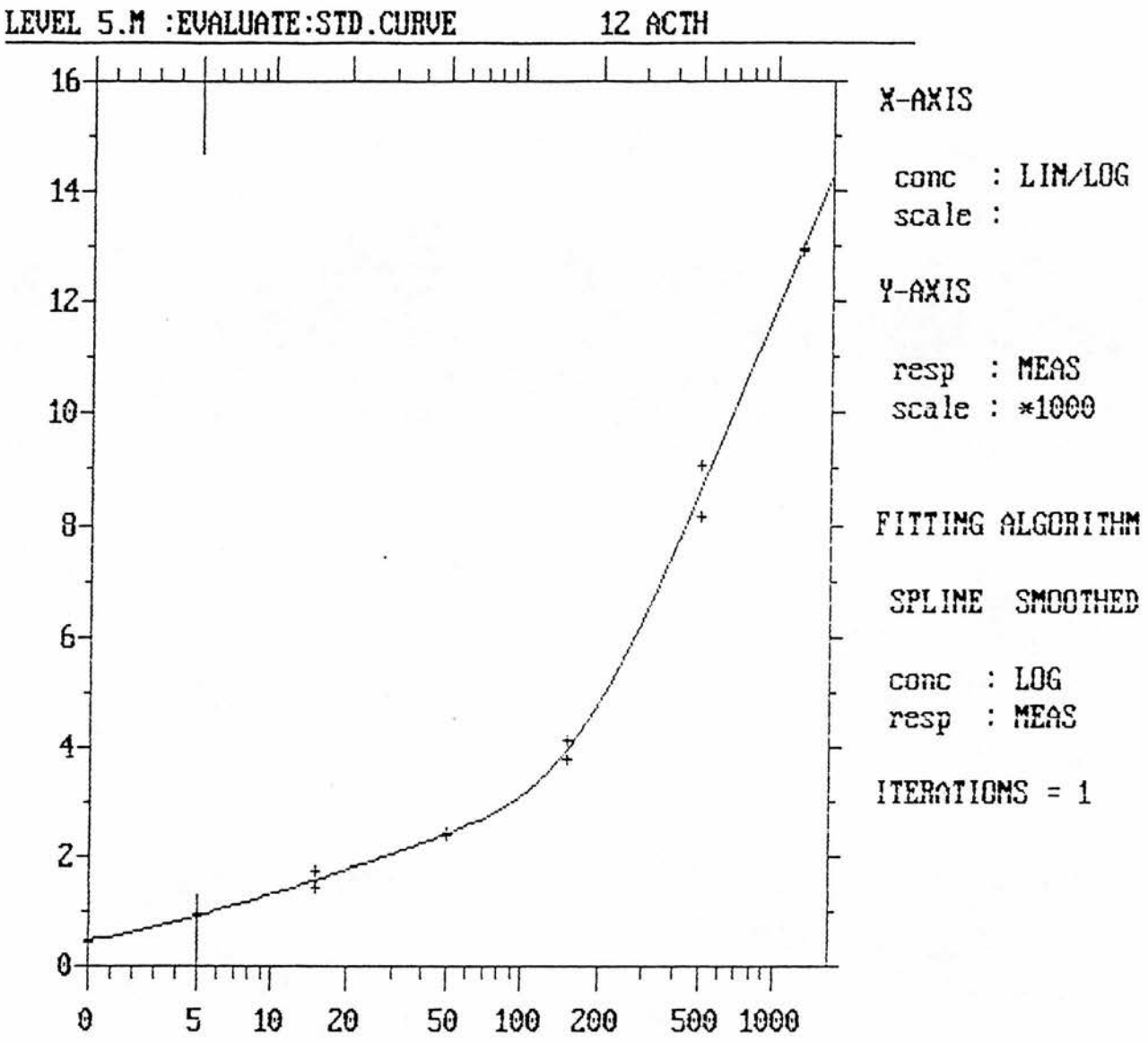


Figure 2.3: A representative ACTH standard curve from the Two-site Immunoradiometric Assay.

The sensitivity of the assay was 0.8 pg/ml and the intra- and inter-assay coefficients of variation were 3% and 5%, respectively.

2.1.3 Corticosterone Radioimmunoassay

Total plasma corticosterone concentrations were measured with an in-house radioimmunoassay. The plasma was diluted 10:1 with borate buffer (163 mM boric acid, 81 mM sodium acetate, 0.5% BSA, pH 7.4) and heated at 75 ^{o}C for 30 min to denature the protein in the samples. A 20 µl aliquot of each of the corticosterone standards (0.625 - 320 mmol/l) or the diluted sample were incubated with a mix of [1,2,6,7 ^{3}H]corticosterone (total counts, 11,000 cpm per tube) and rabbit anti-corticosterone antibody (Dr C. Kenyon, HBP Unit, Glasgow) for 60 min at room temperature. Afterwards, 50 µl anti-rabbit scintillation proximity assay (SPA) reagent was added and the samples incubated overnight. This SPA reagent acts by binding to the first antibody-corticosterone complex, bringing it in close contact with the scintillant and allowing detection of the energy signal. In contrast, the radioactivity of the unbound ligand goes undetected because it is not brought into close enough contact with the scintillant. The samples were quantified in a β-counter and using the Microsoft MultiCalc programme, a standard curve (Figure 2.4) was generated and the data expressed as ng/ml.

The sensitivity of the assay was 1.25 nmol/l and the intra- and inter-assay coefficients of variation were below 7% and 10%, respectively.

A preliminary study was carried out to confirm that our colony of female Sprague Dawley rats possessed a normal corticosterone diurnal rhythm. All the rats exhibited a robust circadian rhythm (Fig 2.4b) which was in agreement with the previously reported range for female rats in the morning and the evening (Carey et al, 1995; Viau & Meaney, 1991). However, in subsequent studies we were unable to consistently achieve these low morning levels. This may have occurred for a number of reasons. Firstly, the levels may simply reflect a lasting effect of the anaesthetic for jugular implantation since it has previously been shown that some anaesthetics

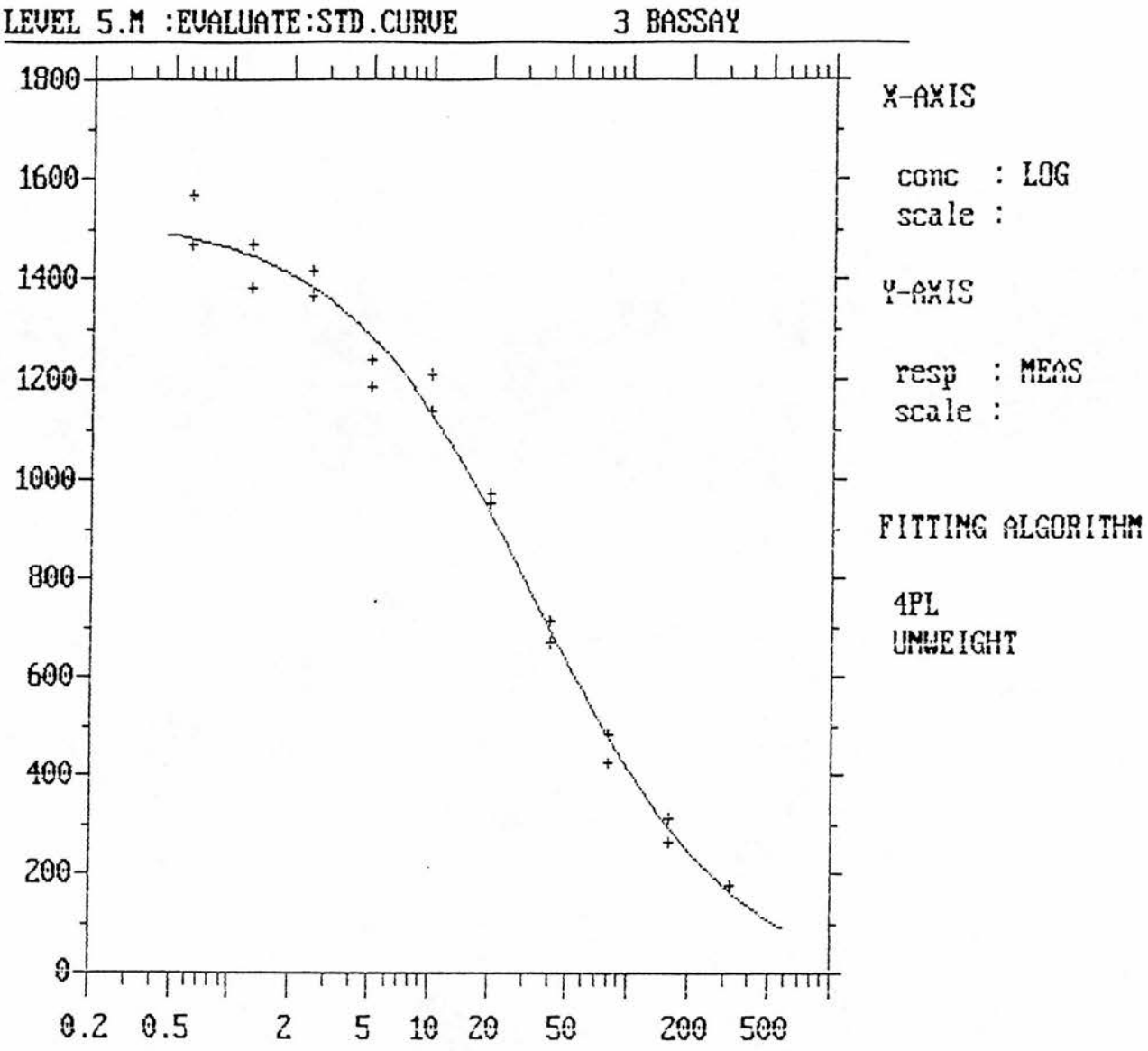


Figure 2.4a: A representative corticosterone standard curve from the corticosterone radioimmunoassay.

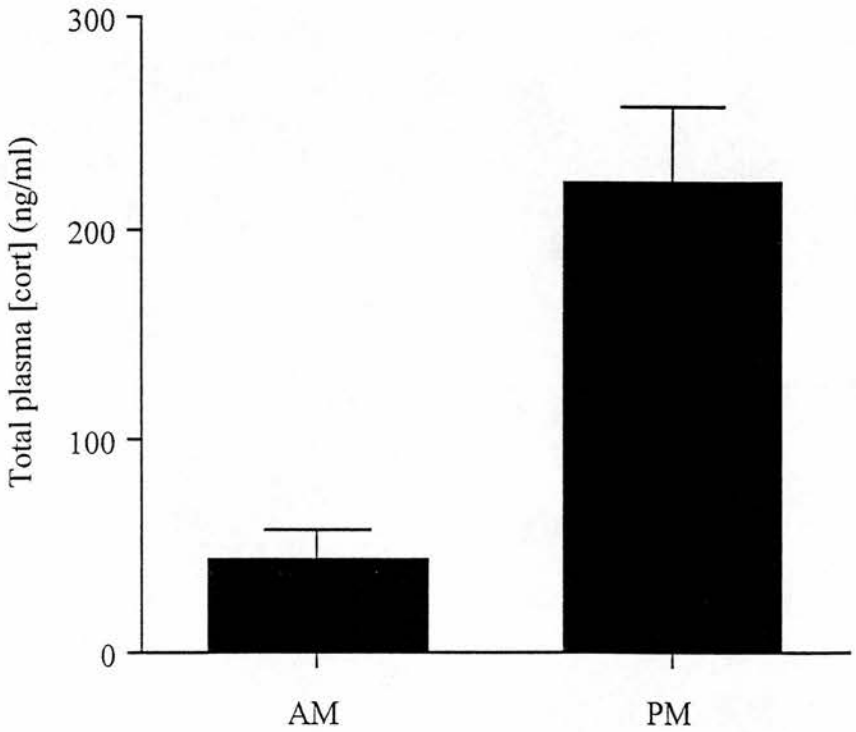


Fig 2.4b: Corticosterone circadian rhythm in virgin female Sprague Dawley rats (n = 6). Animals were fitted with right jugular vein cannula and left to recover from the surgery for 4 days. On the morning of the experiment a jugular venous sampling cannula was connected and the animals left undisturbed for 90 min then a single blood sample (AM) was collected. On the evening of the experiment again the sampling cannula were connected 90 min prior to taking the blood sample (PM). Plasma was assayed for corticosterone. Data expressed as ng/ml (mean ± S.E.M.)

influence the secretion of ACTH secretagogues such as CRF (Sherwood & Fink, 1991) however, our basal ACTH levels fall within the normal range. Secondly, the environment in which the animals were housed may preclude the measurement of low basal corticosterone levels, although on the morning of the experiment the animals were left undisturbed for a minimum of 90 min to allow the levels to return to basal after the connection of the sampling cannulae. It should be noted that the animals were housed singly for 10 days prior to experimentation and this may account in part to the elevated basal values seen.

2.1.4 Corticosterone Binding Globulin Assay

Plasma samples obtained from basal blood collected via indwelling venous cannulae were used to measure CBG levels by the method of Martin *et al.* (1977).

Endogenous steroids were removed by running 20 µl aliquots of plasma in duplicate through Sephadex LH-20 columns and diluting them 50:1 with TEGMD (30 mM Tris, I mM EDTA, 10% glycerol, 10 mM sodium molybdate, and 1 mM dithiothreitol, pH 7.4). 150 µl of diluted plasma was then incubated for 24 h at 4 ^{o}C with 200 nM [1,2,6,7 ^{3}H]corticosterone in the absence or presence of 0.1 mM unlabelled corticosterone, representing total and non-specific binding, respectively.

Separation of the bound from the free corticosterone was achieved by passing the samples through Sephadex LH-20 columns. The elutant was collected into scintillation vials containing 3 ml of Picofluor scintillant and counted on a β-counter. The protein concentrations of the samples were measured colorimetrically (Bio-Rad protein assay kit).

Plasma levels of CBG were expressed as pmol [^{3}H]corticosterone bound/mg of protein.

2.1.5 Enzyme Immunoassay (Magnetic Solid Phase) for 17β-estradiol and progesterone

To determine the plasma concentrations of 17β-estradiol (estradiol) and progesterone achieved in animals s.c implanted with ovarian steroid capsules,

commercially available enzyme immunoassay kits (Biodata Diagnostics, Italy) were used. The estradiol and progesterone assays employ high affinity polyclonal and monoclonal antibodies, respectively, and incorporate a magnetic solid phase separation technique (Abraham, 1969) which eliminates the need for centrifugation. These assays measured total circulating steroid concentrations as they contain a specific agent which displaced the sex steroids from their respective binding proteins.

2.1.5.a Estradiol Asssay

Unextracted plasma, reconstituted estradiol (estra-1,3,5 (10)-triene, 17β-diol) standards (50 - 3000 pg/ml) and internal controls containing a known concentration of estradiol were incubated with an anti-estradiol fluorescein-labelled rabbit polyclonal antibody for 20 min at 37 °C. Afterwards a fixed amount of an estradiol-derivative conjugated to the enzyme, bovine alkaline phosphatase was added and incubated for a further 20 min. This competes for binding to the limited amount of antibody.

The separation reagent containing sheep anti-fluorescein serum bound to magnetic particles was added in excess and incubated for 5 min at 37 °C. Separation of the bound from the free estradiol was achieved by placing the samples on a magnetic rack for 2 min, this causes the magnetic particles to sediment in the tubes. The supernatant was then decanted, a wash buffer added and the particles allowed to resediment for a further 2 min. The decanting, washing and separation steps were repeated. Afterwards the samples were incubated with the enzyme substrate, phenolphthalein monophosphate (PMP) for 30 min at 37 °C. The bovine alkaline phosphatase catalyses the hydrolytic removal of a phosphoryl group from PMP. The enzyme reaction was terminated with the addition of a 'stop' solution, containing a chelating agent, and a purple colour developed. The magnetic particles were then allowed to sediment for a minimum of 10 min prior to the intensity of the colour formed being measured photometrically (Serozyme I Photometer) at 550 nm. The colour intensity was inversely proportional to the amount of estradiol in the sample.

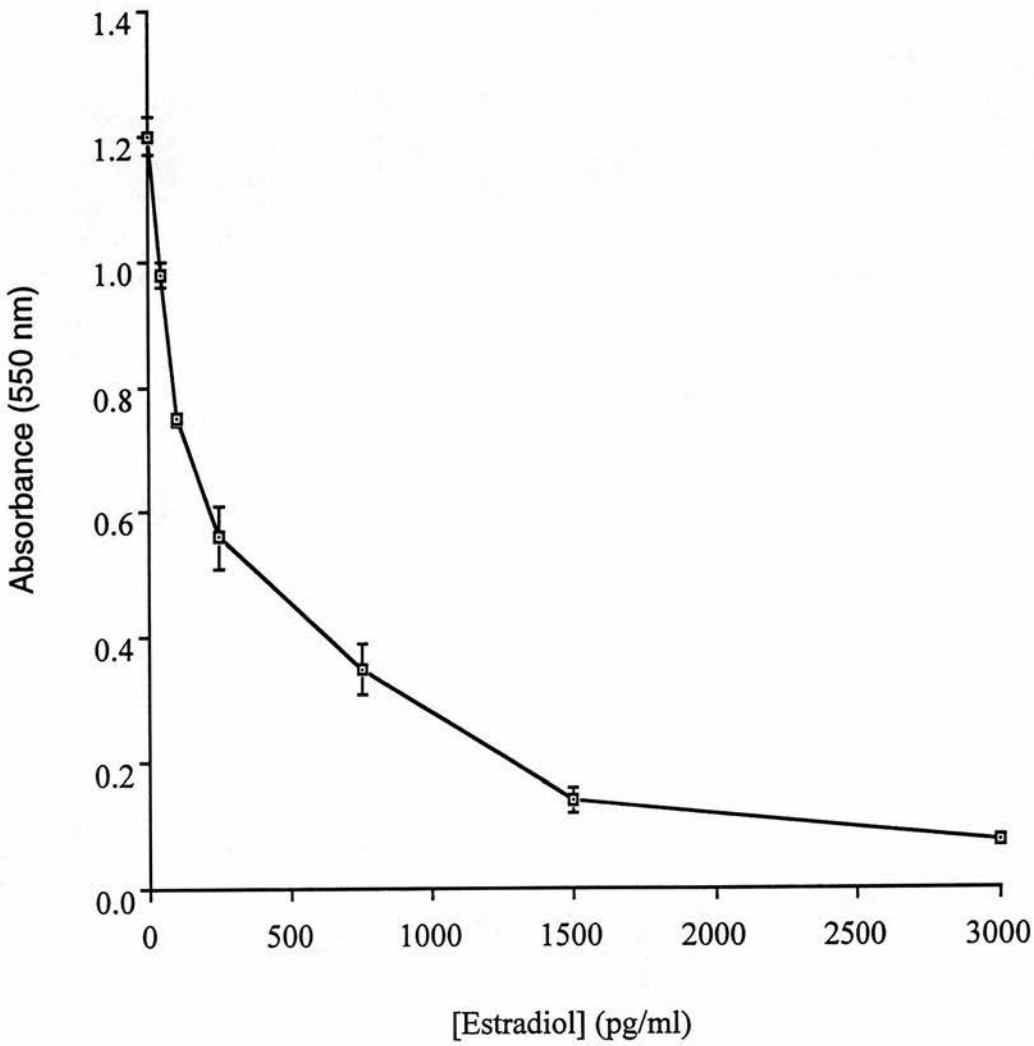


Fig 2.5a: A representative Estradiol Standard Curve from the Enzyme Immunoassay (Magnetic Solid Phase).

The concentrations of estradiol in the plasma samples were determined directly from the standard curve and expressed as pg/ml (Fig 2.5a). The sensitivity of the assay was 0.8 pg/ml and the intra- and inter-assay coefficients of variation were 6% and 7%, respectively.

2.1.5.b Progesterone Assay

The plasma samples from animals treated with steroid-containing capsules were diluted 1 in 5 prior to assay. In previous studies this dilution was found to bring the progesterone concentration within the linear part of the assay standard curve.

Unextracted plasma, reconstituted progesterone standards (0.5 - 40 ng/ml) and internal controls were incubated with fluorescein-labelled progesterone and an anti-progesterone bovine alkaline phosphatase labelled mouse monoclonal antibody for 15 min at 37 ^{o}C. Bound progesterone was separated from the free by the same method used in the estradiol assay. Briefly, following the addition of the sheep antiserum to fluorescein bound to magnetic particles, the samples were incubated for 10 min at 37 ^{o}C and then placed on the magnetic rack for 2 min. The supernatant was decanted, the remaining sediment washed and the magnetic particles resedimented for a further 2 min and the resulting supernatant discarded. The enzyme substrate, PMP was added and the samples incubated for 15 min at 37 ^{o}C. The reaction was terminated by the addition of the 'stop' solution and the magnetic particles sedimented for 10 min, prior to photometric quantification. As with the estradiol assay, the concentration of endogenous progesterone was inversely proportional to the colour intensity. From the standard curve the progesterone concentrations in the plasma samples were determined and the data expressed as ng/ml (fig 2.5b).

The sensitivity of the assay was 0.48 ng/ml and the intra- and inter-assay coefficients of variation were 4% and 8%, respectively.

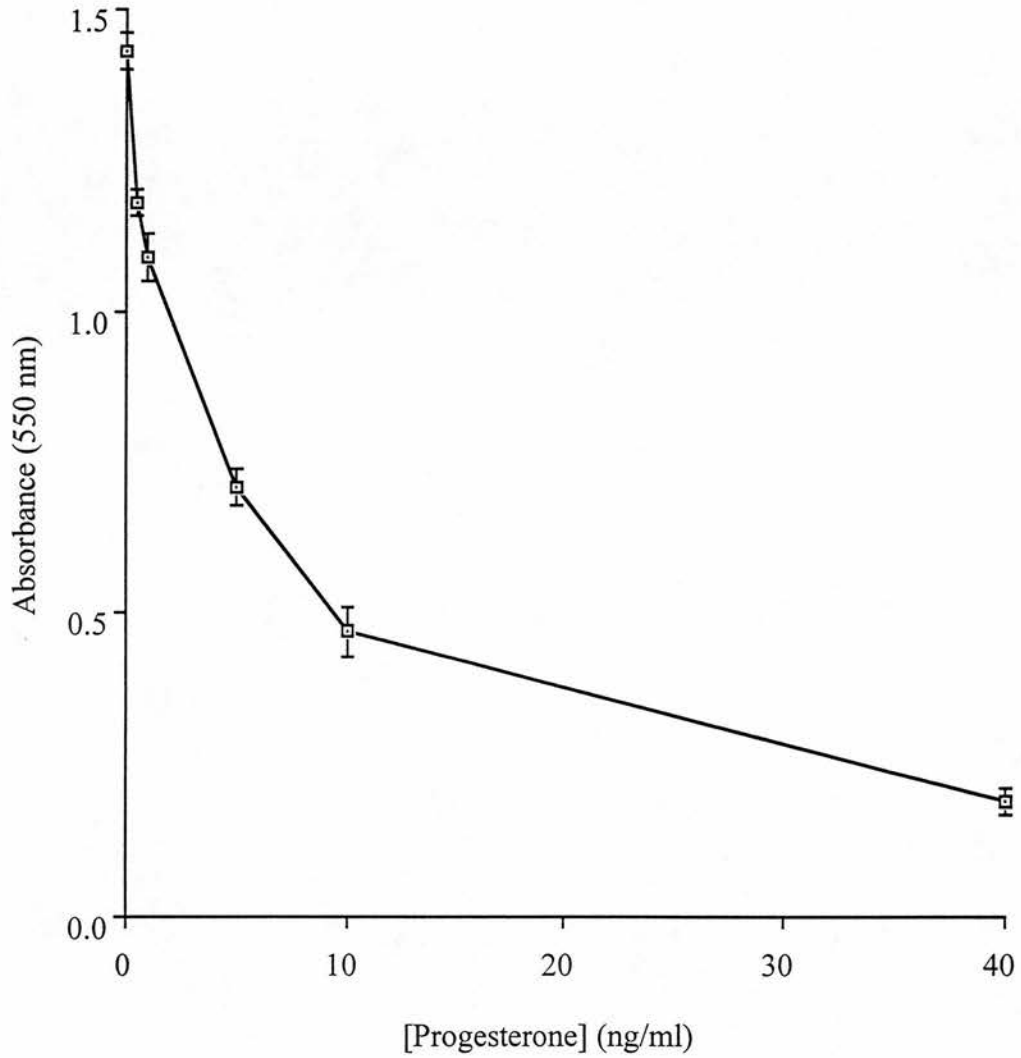


Fig 2.5b: A representative Progesterone Standard Curve from the Enzyme Immunoassay (Magnetic Solid Phase).

2.1.6 *In Vitro* Pituitary Segments

Day 10, 16 and 20 pregnant and female virgin rats were decapitated and the pituitary removed and the neuro-intermediate lobe carefully dissected away. The anterior pituitaries were then washed in Dulbecco's Modified Eagle's medium, (Gibco-BRL), buffered with 25 mM (HEPES) to pH 7.4 containing 0.25% bovine serum albumin (BSA) (Sigma, RIA grade V), and weighed. Using a dissecting microscope and a straight-edged blade, the anterior pituitaries were cut into eight equal sized segments and placed in 24-well cluster plates (Costar), one segment per well, containing 1 ml of DMEM and incubated for 60 min at 37 ^{o}C. They were then transferred to fresh cluster plates containing 250 µl per well of the phosphodiesterase inhibitor, 3-isobutyl-1 methylxanthine (IBMX) (0.5 mM) and incubated for a further 15 min at 37 ^{o}C. Thereafter, each segment was challenged with 250 µl of either human CRH (hCRH) (final concentration 10 nM; Peninsula) or vehicle (DMEM) for 10 min at 37 ^{o}C, with the reaction terminated by the addition of 500 µl of ice-cold 0.2N HCl. The cluster plates were sealed with parafilm (BDH) and stored at -70 ^{o}C until trituration and cAMP determination.

2.1.6.a Non-acetylated cAMP Radioimmunoassay

Following two rounds of freeze-thawing, pituitary segments were triturated with gauge 26 needles, to break up the tissue, allowing the determination of total cAMP levels (both intracellular and extracellular) by the method of Dafau *et al.* (1973).

Following centrifugation at 2,500g for 10 min the resulting supernatant was used in the assay. Duplicate 50 µl aliquots of cAMP standards (ranging from 12.5 - 3200 fmol/50 µl) and samples, along with 4 tubes each for total (1:1 mix of 0.2 N HCl and DMEM) and non-specific (1 mM cAMP) binding were incubated with 100 µl of specific rabbit anti-cAMP antibody (cAB4), 50 µl of [^{125}I]cAMP (14 000 cpm/tube) and 10 µl of the second antibody mix (70% anti-rabbit IgG, 10.5% non

immune rabbit (NRS) and 19.5% assay buffer (50 mM sodium acetate, pH 6.0 with 0.25% BSA), overnight at 4 ^{o}C.

The following morning 700 µl of ice-cold PEG-SERUM (20% polyethylene glycol, 10% horse serum, 0.9% saline, and 50 mM Na acetate) was added and the samples centrifuged at 2500g at 4 °C for 25 min. The supernatant was then decanted and the pellet counted in a γ-counter. The NSB was subtracted from the standards/samples and from the standard curve the total cAMP accumulation in the samples was determined and expressed as nmol/well.

2.1.7 Acutely Dispersed Anterior Pituitary Cells

Day 21 pregnant and virgin rats were quickly decapitated and the anterior pituitaries removed and transferred into DMEM. After the removal of the neuro-intermediate lobe, each pituitary was finely chopped with a straight-edged scapel blade and then pooled together with other pituitaries within each group. The pituitary pieces were then incubated with DMEM containing 0.25% trypsin (TRL), 10 mg/ml DNAse 1 (Sigma) and 0.25% BSA (MILES) for 20-30 min, to break up the tissue, and every 5 min the pituitary pieces were gently dispersed with a 5 ml pipette, then to inactivate the trypsin, DMEM containing 0.5 mg/ml lima bean trypsin inhibitor (Sigma), 100 kallikrein units (KU) aprotinin (Sigma) and 0.25% BSA was added. The remaining tissue fragments were then triturated by a 5 ml pipette tip fitted with a cut-down 1 ml pipette tip for approx. 10 min. The resulting cell suspension was filtered through a 100 µm nylon mesh and diluted with an equal volume of DMEM containing 0.25% BSA, then centrifuged at approx. 600 g for 5-10 min to pellet the cells. The supernatant was carefully removed and the cell pellet resuspended in 1 ml of DMEM. This was then made up to 10 ml with DMEM containing 100 KU aprotinin and the suspension gently rotated for 2 h to allow the cells to equilibrate. Then the suspension was recentrifuged, the supernatant removed and the pellet resuspended in 1 ml of DMEM containing 0.25% BSA. An aliquot of the suspension was counted on a haemocytometer and the number of cells/ml calculated. The suspension was then diluted down to give 2 x 10^5 cells/tube.

These anterior pituitary cells were then exposed to either increasing concentrations of hCRH: 0.1, 1 & 10 nM or vehicle (DMEM) for 10 min at 37 ^{o}C. Each concentration was assayed in quadruplicate and the reaction was started with the addition of the cells. To terminate the stimulation ice-cold 0.2 N HCl was added. The tubes were then sealed with parafilm and stored at -70 ^{o}C until assayed for cAMP determination.

2.1.7.a Acetylated cAMP Radioimmunoassay

This assay is identical to the non-acetylated cAMP assay except for an acetylation step. The acetylation of cAMP increases the sensitivity of the assay approx. 100 fold. Therefore, it is possible to measure small changes in cAMP production more accurately. We used this assay to measure the production of cAMP in acutely dispersed anterior pituitary cells challenged with hCRH.

A mix of 1:2.5 (v/v) of fresh acetic anhydride:triethylammonium was prepared at room temperature. While rapidly vortexing a 10 µl aliquot was added to tubes containing 250 µl of either the cAMP standards (0.78-400 fmol/50 µl) or samples which were then immediately placed on ice.

The assay then followed the same protocol as for the non-acetylated assay (see above). Throughout the assay the tubes were kept on ice as acetylated cAMP is highly unstable at room temperature.

The cAMP concentrations in the samples were quantified in a γ-counter and determined in the same way as for the total cAMP accumulation in pituitary segments (see above), and then expressed as pmol/2 x 10^5 cells.

2.2 In Vivo Studies

2.2.1 Animal Maintenance

Female Sprague Dawley rats (230-250g) (Bantin and Kingman, UK) were routinely used for *in vivo* studies. The animals were housed in groups of five per cage

under standard laboratory conditions: controlled lighting (lights on:lights off, 07 00:19 00 h), ambient temperature 22 °C with food and water *ad libitum*, on arrival at the central animal facility, University of Edinburgh. They were then allowed a period of at least two weeks to acclimatize to their new surroundings prior to experimentation.

The animals were handled daily for a minimum of a week prior to an experiment in order to reduce non-specific stress effects occurring during experimentation.

2.2.2 Surgery

2.2.2.a Chronic jugular cannulation

Under halothane:nitrous oxide anaesthesia pregnant and virgin rats were implanted with a jugular cannula for blood sampling. Using sterile procedures the right jugular vein was carefully exposed and a silastic catheter (ID 0.5 mm; OD 1 mm), containing sterile heparinised saline (heparin 20U/ml 0.9% saline), was inserted for a distance of 3 cm so that the tip lay within the right atrium of the heart. To check that the cannula was in place a small volume of blood was drawn back prior to the cannula being secured in place with suture thread. The cannula was then exteriorised at the back of the neck, fixed in placed with a strip of tape which was sutured to the skin and the cannula was sealed with a plug. The animals were then allowed a minimum of three days to recover.

2.2.2.b Subcutaneous Silastic Capsule Implantation

Under halothane:nitrous oxide anaesthesia and using sterile procedures female virgin rats were implanted subcutaneously with silastic capsules containing ovarian steroids (modified from Bridges, 1984).

Silastic tubing (ID 0.2 cm; OD 0.3 cm) was cut to a length of either 1.5 cm or 3 cm for estradiol and progesterone capsules, respectively, and sealed at one end with silicone sealant and autoclaved. Thereafter, sterile conditions were used.

17β-estradiol was dissolved in vehicle oil (89.7% arachis oil, 10% benzyl alcohol, 0.3% cresol) to a concentration of 15 mg/ml and used to fill the estradiol capsules, and crystalline progesterone was used to fill the progesterone capsules. All capsules were then sealed, washed in absolute ethanol and incubated in phosphate-buffered saline for a minimum of 24 h prior to implantation.

One 17β-estradiol or vehicle capsule was implanted on day one of surgery and seven progesterone or empty capsules were implanted two days later. Then, after approximately two weeks a jugular cannula was inserted to enable serial blood sampling. As previously mentioned the animals were then allowed a minimum of three days to recover prior to experimentation.

2.3 In Situ Hybridisation Histochemistry

In situ hybridisation histochemistry is a technique which allows the precise localisation and identification of individual cells containing specific nucleic acid sequences. It involves the hybridisation of single-stranded nucleic acid molecules recognising one another and forming hydrogen bonds between complementary base pairs. In the early studies in situ hybridisation was used primarily to detect DNA targets and amplified ribosomal (r)RNA genes within cell nuclei (Gall & Pardue, 1969; John *et al.* 1969; Buongiorno-Nardelli & Armaldi, 1970). However, over time the sensitivity of the technique has increased, thus allowing the detection of specific messenger RNAs (mRNAs) expressed at very low levels in cells.

The stability of the hybrids formed, whether they are DNA-DNA, RNA-RNA or DNA-RNA duplexes, is governed by several factors, such as temperature: increasing temperature promotes dissociation of the duplexes; ionic strength: the presence of salt acts in two ways to stabilise the nucleic acid duplexes. Firstly, by neutralising the electrostatic repulsive forces between the negatively charged phosphate groups on opposing strands and secondly by decreasing the solubility of the bases and thus strengthening the hydrophobic interactions between them; % of guanosine/cytosine (GC) base pairs: duplexes with high GC content are more stable because of the increased number of hydrogen bonds they possess; and probe length:

long complementary sequences, which contain more hydrogen bonds, allow the orderly stacking of bases and thus result in less base pair mismatch occuring.

Choosing the correct probe for in situ hybridisation is a critical decision. There are three main classes of probe 1) complementary DNA (cDNA) probes: cDNA probes are double-stranded, relatively easy to label and use, have high specific activity and thus give good amplification of the signal. However, the double strands have to be denatured prior to application to the tissue sections and reannealing of the strands has been shown to occur, thus significantly reducing the amount of probe available for hybridisation (Cox *et al.*, 1984); 2) oligonucleotide probes: these are short (normally 15-50 base pairs long) single-stranded DNA oligonucleotides which are quick and easy to produce. Their short probe length allows better penetration of the tissue sections, however, it also means that less stringent hybridisation conditions have to be employed leading to a loss of specificity; 3) RNA probes (riboprobes): these are single-stranded RNA molecules produced from a cloned cDNA that is introduced into a specifically designed plasmid transcription system.

We chose to use riboprobes for our *in situ* hybridisation studies because there is no problem about reannealing since they are single-stranded, the transcription reaction produces a labeled probe of a fixed length and high specific activity which forms a highly stable hybrid. These probes have a tendency to hybridise to non-specific sites in the tissue sections, however, pre- and post-hybridisation techniques are employed to reduce this.

To be able to determine the specificity of the hybridisation signal of the probe, termed the antisense probe, a heterologous probe, termed the sense probe, which has a similar length, GC content and specific activity as the antisense probe was included in the *in situ* hybridisation studies. The lack of signal with the sense probe verifies that probe binding is a result of its base sequence and not its physical properties.

2.3.1 Plasmid Linearisation

2.3.1.a Rat Glucocorticoid receptor (rGR)

The pGEM4 plasmid containing a 674 base pair (bp) Pst1/EcoR fragment of the rGR cDNA, corresponding to the steroid binding domain, (base pair 1734-2364) was linearized by incubation with the restriction endonucleases, Ava 1 or EcoR1 to generate an antisense or sense DNA fragment, respectively, for 1-2 h at 37 ^{o}C (Seckl *et al.*, 1990). A kind gift from Dr Yau, Edinburgh.

2.3.1.b Rat Mineralocorticoid receptor (rMR)

The pGEM4 plasmid containing a 513 bp EcoR1 fragment of the rMR cDNA, corresponding to part of the steroid binding domain and 3' untranslated region, (base pair 1692-2205) was linearized by incubation with either Hind III or EcoR1 to generate an antisense or sense DNA fragment, respectively, for 1-2 h at 37 ^{o}C (Seckl *et al.*, 1990). A kind gift from Dr Yau, Edinburgh.

2.3.1.c Rat Corticotropin-releasing factor

The Bluescript plasmid containing a 540 bp fragment of the rat corticotropin-releasing factor (rCRF) cDNA (base pair 1525-2065) was linearized by incubation with either Hind III or EcoR1 to generate the antisense or sense DNA fragments, respectively, for 1-2 h at 37 ^{o}C. A kind gift from Dr Diaz.

2.3.1.d Rat Arginine Vasopressin (rAVP)

The pGEM3 plasmid containing a 460 bp Hind III/EcoR1 fragment of the rat arginine vasopressin (rAVP) cDNA (base pair 7-467) was linearized with either EcoR1 or Hind III to generate the antisense or sense DNA fragment, respectively, for 1-2 h at 37 ^{o}C. A kind gift from Dr Sherman.

To confirm that each plasmid had been cut correctly an aliquot of the reaction mix in loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF & 15% ficoll 400) was run on a 1.5% agarose gel containing the alkylating agent ethidium bromide at 10 mA and visualised under UV light.

2.3.2 Phenol/chloroform Extraction of the Plasmid

To clean the DNA fragments an equal volume of phenol/chloroform was added, the mix vortexed then spun at 13 000 rpm for 2 min and the upper aqueous layer, containing the DNA, collected into a fresh RNAse-free eppendorf. The remaining lower organic layer was back-extracted i.e. 50 µl of (depc-H_2O) was added and the vortexing and spin steps repeated. The resulting top layer was taken into the fresh eppendorf and an equal volume of chloroform:isoamyl (24:1) was added. It was then vortexed and centrifuged. The upper layer was taken into another fresh eppendorf and the remaining organic layer back-extracted again. The DNA fragment was ethanol-precipitated with 0.1 volumes of 3 M Na acetate and 2.5 volumes of ethanol. The mix was vortexed, placed on dry ice for 10 min then centrifuged for 15 min and the supernatant removed. The resulting DNA pellet was carefully washed in 70% ethanol, vortexed and spun for 5 min. The supernatant was taken off and the pellet dried in the Speedvac at a low speed for 15 sec. The DNA pellet was resuspended in depc-H_2O and to check that there was no contamination a 1 µl aliquot was run on a 1.5% agarose gel. The remaining template was aliquoted into fresh eppendorfs at a concentration of 1 mg/ml and stored at -20 ^{o}C.

2.3.3 ^{35}S-UTP-labelled cRNA Probes

All of the linearized cDNA fragments were incubated with excess cold nucleotides (10 mM ATP, CTP & GTP), 200 mM DTT , a RNase inhibitor, 50 mM ^{35}S-UTP (specific activity 40 mCi/ml) 1 µl of the specific RNA polymerases in the presence of 5x transcription buffer to give a final volume of ~ 10 µl.

2.3.3.a rGR cDNA

The rGR cDNA was incubated for 60 min with the RNA polymerases, T7 and SP6 to produce antisense and sense transcripts, respectively. The incubation was carried out at 37 ^{o}C for T7 and 40 ^{o}C for SP6 polymerases.

2.3.3.b rMR cDNA

The rMR cDNA was incubated with SP6 to generate the antisense and T7 to transcribe the sense transcript. This is opposite to that for the rGR because the polycloning site in the pGEM4 plasmid is in the opposite orientation to pGEM3.

2.3.3.c rCRH cDNA

To generate antisense and sense single-stranded RNA transcripts the rCRH cDNA was incubated with T3 and T7 RNA polymerases, respectively, for 60 min at 37 ^{o}C.

2.3.3.d rAVP cDNA

The antisense and sense transcripts were generated by incubating the rAVP cDNA with SP6 and T7 RNA polymerases, respectively, for 60 min at 40 ^{o}C (SP6) and 37 ^{o}C for T7 reactions.

Following the above transcription incubations 2 µl of 5x transcription buffer, 1 µl of RNase-free DNase and 8 µl of depc-H_2O were added to each transcription reaction mix and incubated for 10 min at 37 ^{o}C. The reaction was stopped by placing the tube on ice and the reaction volume was made up to 50 µl with depc-H_2O.

To purify the newly synthesised probe the mix was run through a 'Nick' column (Pharmacia), prepared by loading 3 ml of Tris-EDTA (TE) buffer, pH 8.0 to equilibrate the gel bed. The probe was applied to the top of the column followed by 400 µl of TE buffer. The elutant, which contained very little radioactivity as it

contained predominantly free nucleotides, was discarded and a further 600 µl of TE buffer applied to the column. This elutant was collected as it contained the labelled cRNA probe.

To check the incorporation of the ^{35}S-UTP a 1 µl aliquot of the probe was counted in a β-counter (2-5 x10^5 cpm) and to determine if degradation of the probe had occurred 1 µl was run on a 6 M urea:5% polyacrylamide gel at 10 mA. Then, the gel was placed against Hyperβ-max film, (Amersham International) overnight and developed. The remaining probe was stored at -20 ^{o}C for a maximum of a week prior to use for *in situ* hybridisation histochemistry.

2.3.4. Tissue Collection

Day 10, 16 and 21 pregnant and virgin Sprague Dawley rats were decapitated, the brains rapidly removed, placed on tin foil on dry ice and covered with powdered dry ice. Once completely frozen they were stored at -80 ^{o}C.

20 µm coronal brain sections containing either the PVN or the anterior hippocampus were cut using a cryostat at -20 ^{o}C. The former area was identified by staining every third section with pyronin Y (Sigma) and examining under a light microscope. The sections were thaw-mounted onto gelatin and poly-L-lysine coated slides and stored at -80 ^{o}C until used for specific *in situ* hybridisation analysis.

2.3.5 Tissue Fixation

Sections were fixed in 4% (w/v) paraformaldehyde/0.1 M Na phosphate buffer (20 mM NaH_2PO_4, 80 mM Na_2HPO_4, pH 7.4) for 10 min at room temperature. This is an alkylating agent which forms cross-links with nucleic acids and proteins thereby maintaining tissue morphology and retaining target nucleic acids. The fixation step was immediately followed by three washes in 2 x SSC (20 x SSC, Sigma) made up in depc- H_2O for 5 min each.

2.3.6 Prehybridisation

To reduce background hybridisation of the probe all tissue sections were treated with prehybridisation buffer (5M NaCl, 1 M Tris-HCl, pH 7.5, 50x Denhardt's, 250 mM EDTA, 10 mg/ml salmon sperm DNA, 50 mg/ml yeast tRNA). The components of this buffer decrease the non-specific binding to proteins, polysaccharides and nucleic acids in the tissue sections. Sufficient prehybridisation buffer diluted 1:1 with deionised formamide was made up to allow the application of 200 µl to each slide. The tissue sections were drained and dried around the edges with lens tissue prior to the addition of the buffer and placed flat in the hybridisation boxes on 3 MM Whatman paper soaked in box buffer (4 x SSC, 50% deionised formamide in depc-H_2O) and incubated at 50 °C for 2 h.

2.3.7 Hybridisation

Similarly, sufficient hybridisation buffer was made up to apply 200 µl per slide. The mix consisted of 50% deionised formamide, 10 x 10^6 counts/ml of radioactive cRNA probe made up to the final volume with 2 x hybridisation buffer (5 M NaCl, 1 M Tris-HCl, pH 7.5, 50x Denhardt's, 250 mM EDTA, 10mg/ml salmon sperm DNA, 2 g dextran sulphate & 50 mg/ml yeast tRNA). The mix was denatured at 70 °C for 5 min, cooled on ice and 1 M DDT (10 µl/ml of probe) added.

The tissue sections were once again drained and dried around the edges prior to the hybridisation mix being applied. The slides were then placed back in the hybridisation boxes, sealed, and hybridised overnight at 50 °C.

The following day the slides were drained and washed three times in 2 x SSC (NaCl, Na Citrate, Sigma) for 5 min each. They were then exposed to RNase digestion. 200 µl of RNase buffer (5 M NaCl, 1 M Tris-HCl, pH 7.5, 250 mM EDTA) containing 10 mg/ml RNase A (3 µl/ml of buffer), a nuclease which specifically degrades single-stranded nucleic acids, was applied to each slide, which were then incubated at 37 °C for 60 min.

Afterwards the slides were cycled through progressively more stringent washes, beginning with a 30 min wash in 2 x SSC at room temp, followed by two

washes in 0.1 x SSC at 60 °C for 60 min each. The sections were then dehydrated in 50%, 70%, and 90% ethanol in 0.3 M ammonium acetate for 2 min each. They were then allowed to air-dry and placed against Hyperfilmβ-max (Amersham International) for one week.

Following film development (D19, Kodak-Pathe, France) the slides were dipped in autoradiographic emulsion (NTB2, Kodak, USA) and stored in lightproof boxes at 4 °C for 21 days before being developed and counterstained with pyronin Y. Silver grains were counted using a high-power (x40 objective) microscope linked to a computer-based image analysis system (SeeScan). The number of silver grains per neurone was counted. The data were expressed as the average no. of silver grains/neurone in each brain region measured. Background was subtracted, which was always counted over a white matter area as it was low due to the highly stringent conditions used.

2.4 Receptor Autoradiography

2.4.1 Tissue Collection

Whole pituitaries from day 10, day 16 & day 21 pregnant or virgin Sprague Dawley rats were collected following decapitation. Each pituitary was placed on aluminum foil on dry ice and covered with powdered dry ice and then stored at -80 ^{o}C.

Each pituitary was sectioned horizontally at 20 μm in a cryostat at -20 ^{o}C and mounted onto prechilled poly-L-lysine-coated slides and placed in cooled slide boxes. Following cutting, the sections were desiccated: open slide boxes were placed in a desiccator jar which had previously been cooled to 4 °C and contained silica pellets (Sigma). The air was then evacuated from the jar using a vacuum pump and the slides were allowed to desiccate overnight at 4 °C. The following morning the slide boxes were quickly sealed with moisture-resistant adhesive tape and stored at – 20 °C until use.

From each pituitary three slides were collected: two for total and one for non-specific binding. Each slide contained four pituitary sections.

2.4.2 CRF Receptor Autoradiography

Slides were preincubated for 20 min at room temperature in assay buffer (50 nM Tris/HCl , pH 7.4, 10 mM $MgCl_2$, 2 mM EGTA, 0.1 mM bacitracin (Sigma, Germany), aprotinin (100 KIU/ml, Trasylol, Bayer, Germany), and 0.1% BSA (RIA-grade V, Sigma, Germany) and then carefully dried round the edges with tissues. Subsequently, they were incubated in fresh buffer containing 0.2 nM ^{125}I-ovine CRF (specific activity 2200 Ci/mmol, Dupont, Germany) for 60 min at room temperature. Non-specific binding was determined in the presence of 10^{-6} M unlabelled ovine CRF (Sigma, Germany). The sections were then briefly rinsed and washed three times for 2 min in BSA-free ice-cold buffer,dipped in double-distilled H_2O and dried in a stream of cold air. The slides were then exposed to Agfascopic Video 5B film for 22 days at 4 °C.

Analysis and quantification of the autoradiographs were carried out microscopically (objective x10; additional magnification x1.6) using a computer-based image analysis system (Joyce-Loebl MicroMagiscan).

The optical density was measured within a 100 μm^2 frame over each section. Total binding was measured over two different areas for the anterior pituitary and one area each for the intermediate and the posterior pituitary gland. Non-specific binding was measured over three different areas since the specific pituitary lobes were not distinguishable. Background optical density was determined in two areas of the film off the tissue for each slide and the mean subtracted from the tissue optical density. The mean optical density was calculated for each rat pituitary lobe, the mean non-specific binding subtracted and the group mean calculated.

2.5 Statistical Analysis

Data from all *in vitro* assays and *in situ* hybridisation histochemistry studies were analysed by Analysis of Variance followed by Newman-Keuls Student's test,

except the cAMP accumulation data which was analysed by Student's t test and the 11β-HSD1 conversion assay which was analysed by Kruskal-Wallis followed by Mann-Whitney U test..

All *in vivo* studies were analysed by Two way Analysis of Variance for repeated measures followed by Newman-Keuls Student's test unless otherwise stated. Significance was set at $p < 0.05$ and values were expressed as mean ± S.E.M.

CHAPTER 3

An attenuated HPA axis stress response in pregnancy

3.1 Introduction

The activity of the HPA axis can be altered by several physiological and pathophysiological conditions that challenge the internal homeostasis of the organism. Aging (Hatzinger *et al.*, 1996) and a number of psychiatric disorders, including depression (Holsboer & Barden, 1996), anorexia nervosa (Gold *et al.*, 1986), panic anxiety (Gold *et al.*, 1988b) and obsessive-compulsive disorder (Insel *et al.*, 1982) have been associated with chronic activation of the HPA axis as reflected by enhanced CRF secretion and elevated basal plasma corticosterone concentrations. Alternatively, conditions such as seasonal affective disorder or the postpartum period have been associated with decreased CRF secretion and therefore a hypoactivity of the HPA axis (Vanterpool *et al.*, 1991; Chrousos & Gold, 1992).

The reproductive state of the organism also appears to have profound effects on the responsiveness of the HPA axis. In lactation the basal circulating levels of both ACTH and corticosterone have been shown to be increased (Voogt *et al.*, 1969). An elevation of the morning corticosterone levels and a slight reduction in the peak evening levels has been reported (Stern *et al.*, 1973), resulting in a loss of the normal corticosterone circadian rhythm. A recent detailed study by Atkinson & Waddell (1995) confirmed the previous results. It was found that while corticosterone lost its natural rhythm, due to a decrease in peak levels, ACTH maintained a normal rhythm with elevation in both trough and peak levels. This dissociation between plasma ACTH and corticosterone levels postpartum has been proposed to reflect changes in the circulating ovarian steroid enviroment. During lactation the plasma levels of estrogen are relatively low compared with those in pregnancy (Taya & Greenwald, 1982). Estrogen has been shown to increase adrenal responsiveness, although the mechanism underlying this effect is unknown. Thus the fall in plasma estrogen

concentrations postpartum may result in reduced adrenal sensitivity to ACTH (Kitay *et al.*, 1965) and the relative differences in circulating levels of ACTH and corticosterone.

Furthermore, the classical stress response of the pituitary-adrenal axis appears to be blunted in lactation (Stern *et al.*, 1973). In response to a number of stressors including physical (Altemus *et al.*, 1995; Walker *et al.*, 1995), noise stress (Windle *et al.*, 1997) and exposure to ether (Walker *et al.*, 1992) the ACTH and consequently the corticosterone responses in both humans and rodents are significantly reduced. Several *in situ* hybridisation studies have revealed changes in gene expression occurring in the hypothalamus of lactating rats exposed to an acute stress. In response to an intraperitoneal (i.p.) injection of hypertonic saline (1.5 M) virgin control animals showed a rapid induction in the expression of CRF and AVP mRNA's in the parvocellular PVN. In contrast, the stimulation was completely abolished during lactation (Lightman & Young III, 1989). However, removal of the pups for a minimum of two days resulted in a return of the normal responsiveness of the CRF mRNA to stress (Lightman & Young III, 1989; Stern & Levine, 1972).

Pregnancy may also be seen as a perturbation of the internal environment of the mother; however until recently most of the reports were concerned with effects of pregnancy on the basal activity of the HPA axis. In rats, increased corticosterone levels and the maintenance of a diurnal rhythm have been reported (Dupouy *et al.*, 1975; Ogle & Kitay, 1977; Atkinson & Waddell, 1995). In addition, a concomitant decrease in the plasma immunoreactive ACTH concentrations was found and a loss of its circadian rhythm with progressing pregnancy. Again this dissociation between plasma levels of these hormones may reflect the changes in the relative estrogen plasma levels. During pregnancy the high estrogen levels (Shaikh, 1971) may increase the sensitivity of the adrenal gland to ACTH (Kitay *et al.*, 1965).

A recent collaboration between our group and R. Landgraf's group in Munich, Germany was carried out to determine whether the hyporesponsiveness of the HPA axis to an acute stress, seen in lactation, was established in pregnancy (Neumann *et al.*, 1998). Wistar rats at various stages of pregnancy, which had previously been fitted with jugular cannulae, were exposed to a mild emotional stressor, the elevated

plus-maze (EPM) (Liebsch *et al.*, 1995) followed by a moderate physical/emotional stressor, 90 sec of forced swimming (FS) (Abel, 1994) and their neuroendocrine responses compared with virgin rats. It was found that from day 15 of pregnancy there was a significant reduction ($p < 0.01$, two way ANOVA for repeated measures) in the peak ACTH and corticosterone secretory responses compared to virgin and day 10 pregnant rats.

It is well documented that the responsiveness of the HPA axis varies between rat strains. The Fischer (F344/N) rat strain shows an elevated HPA responsiveness to stress, which is proposed to be mediated through impairment of the glucocorticoid feedback signal. On the other hand the HPA axis of the Lewis rat strain is hyporesponsive to acute stress, which was originally proposed to occur at the level of the parvocellular CRF neurone itself (Sternberg *et al*, 1992), however, recently Rivest & Rivier (1994) provided evidence that this hyporesponsiveness may reflect a defect in the afferent signalling to these neurones.

It is also well known that stressors which may be physical or psychological activate different afferent pathways to the PVN neurones. Thus unlike Hans Seyle's (1936) concept of a 'general stress response' it is now accepted that there is stress-specific activation of PVN neurones leading to the potential for differences in the stress response to occur (Harbuz et al., 1994; Harbuz & Lightman, 1989) .

Thus, we were interested in demonstrating that this phenomenon of an attenuated HPA axis stress response occurred during pregnancy, as demonstrated by Neumann *et al.* In this study we used Sprague Dawley rats and 20 min of restraint stress, a primarily psychological stressor.

3.2 Material and Methods

Individually caged pregnant and virgin animals had a silastic jugular cannula inserted by the method described in detail in Chapter 2. The experiment was carried out three to four days following surgery to allow the animals to recover.

On the morning of the experiment, sampling cannulae attached to 1 ml syringes filled with sterile heparinised saline (20 IU/ml 0.9% saline) were connected between 07.00 and 08.00 h and the animals left undisturbed for 90 min. Following

the collection of basal blood samples each animal was placed in a clear perspex restraining tube (ID 5 cm, tube length 20 cm) for 20 min. Afterwards, they were returned to their homecages and at 5, 15, 30 and 60 min post-stress blood samples were collected. For each blood sample a volume of 0.3 ml (ACTH and corticosterone measurements) was taken and immediately replaced with an equal volume of sterile saline. All blood samples were collected into EDTA-coated tubes on ice and centrifuged at 3000 g for 5 min. The plasma was aliquoted (150 µl for ACTH and 50 µl for corticosterone) and stored at -70 ^{o}C until assayed by sensitive and selective assays. For details see Chapter 2.

At the end of the experiment the animals were killed by an intravenous overdose of anaesthetic (Sagatal) (600 µl/animal)and their pregnancy status checked.

3.3 Results

Two way ANOVA for repeated measures (RM) of the plasma ACTH and corticosterone concentrations in both groups demonstrated a significant interaction between time and group ($p < 0.001$); all other specific comparisons were derived from Newman-Keuls t test ($p < 0.05$).

The basal concentrations of ACTH did not differ significantly between day 21 pregnant ($n = 8$) and virgin rats ($n = 8$), (12.9 ± 5.0 vs. 17.5 ± 4.2 pg/ml, respectively). Following exposure to 20 min of restraint plasma ACTH levels significantly increased in both groups ($p < 0.05$, 12- and 14-fold, pregnant and virgin, respectively, at 5 min). However, the magnitude of the ACTH response to the restraint stress was significantly less in the pregnant animals compared to virgin controls at 5, 15 and 30 min post-stress (Fig 3.1a, $p < 0.05$). At 60 min post-stress the plasma ACTH levels had returned to basal concentrations.

Basal plasma corticosterone concentrations were not significantly different between the day 21 pregnant and virgin groups (131.5 ± 15.1 vs. 111.8 ± 45.2 ng/ml, respectively) and following the restraint stress corticosterone levels reached a

maximum 15 min post-stress (5- and 8-fold, $p < 0.05$, pregnant and virgin, respectively) in both groups. As with ACTH, the magnitude of the corticosterone response to the stress in the day 21 pregnant animals were significantly less at both 5 and 15 min post-stress (Fig 3.1b, $p < 0.05$). At 60 min post-stress the plasma corticosterone levels had returned to baseline levels in both groups.

3.4 Discussion

The results from this study are in agreement with the observations of Neumann *et al* (1998) that the hyporesponsiveness of the HPA axis to an acute stress is established during pregnancy. In addition, our finding suggest that this phenomenon is independent of the type of stress or the rat strain used.

In this study we were able to show a robust increase in plasma levels of ACTH and corticosterone in response to 20 min of restraint in both pregnant and virgin rats. This is in contrast to the finding in lactating rats where following hypertonic saline i.p. there was no significant rise in the plasma corticosterone levels (Lightman & Young III, 1989). However, in that study the basal corticosterone levels in lactating rats were already higher than in the virgin controls, although not significantly so, This fact may account for the absence of a response to the stress and in addition the ACTH response was not measured. We found the peak plasma ACTH and corticosterone responses were significantly reduced by 42% and 20%, respectively, on day 21 of pregnancy which is comparable to the attenuations previously seen by Neumann *et al* (1998) using physical/emotional stressors. These result could simply reflect an enhanced metabolic clearance of ACTH and corticosterone during pregnancy; however, a study by Waddell & Atkinson (1994) clearly demonstrated, at least in the case of corticosterone, that the clearance rate did not differ between pregnant and nonpregnant animals. The ACTH response to the restraint stress exhibited a longer lasting suppression than the corticosterone response. This apparent dissociation between the ACTH and corticosterone response may suggest that during pregnancy the adrenal gland becomes highly sensitive to ACTH. Indeed, estrogen has been shown to increase adrenal responsiveness to ACTH (Kitay *et al.*, 1965).

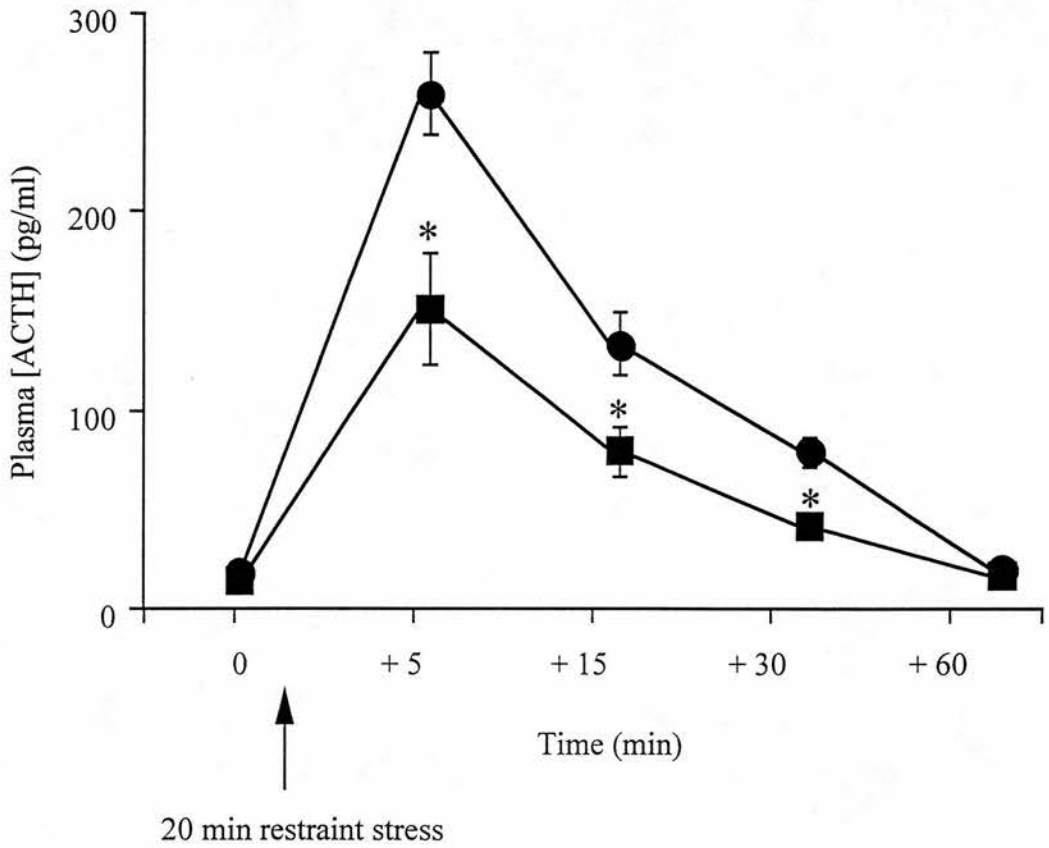


Fig 3.1a: The effect of 20 min restraint stress on plasma ACTH levels in pregnant and virgin rats. Basal blood samples (time 0) were collected from day 21 pregnant (■, n = 8) and virgin (●, n =8) rats. Immediately after, each animal was restrained for 20 min and blood samples collected 5, 15, 30 & 60 min after the end of the stress. Two way ANOVA for RM followed by Newman-Keuls student's test: * $p < 0.05$ vs. virgin group at 5, 15 & 30 min post-stress.

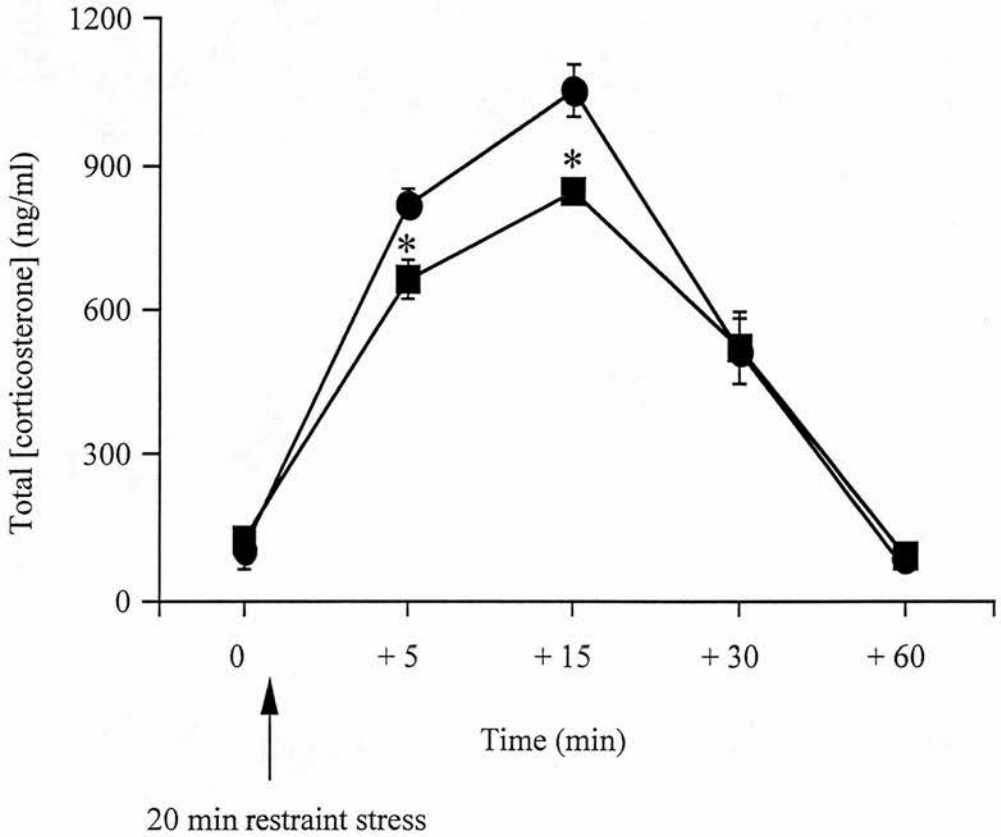


Fig 3.1b: The effect of 20 min restraint stress on plasma corticosterone levels in pregnant and virgin rats. Basal blood samples (time 0) were collected from day 21 pregnant (■, n = 8) and virgin (●, n = 8) rats. Immediately after, each animal was restrained for 20 min and blood samples collected 5, 15, 30 & 60 min after the end of the stress. Two way ANOVA for RM followed by Newman-Keuls student's test: * $p < 0.05$ vs. virgin group at 5 & 15 min post-stress.

The mechanism(s) underlying the suppression of the stress response in pregnancy is/are not known. Several studies have examined the suppression of the responsiveness of the HPA axis during lactation. Interestingly, removal of the pups from the mother for a minimum of two days results in the return of the normal stress responsiveness of the HPA axis (Lightman & Young III, 1989; Stern & Levine, 1972) leading to the suggestion that either suckling *per se* and/or an olfactory stimulus from the pups may be responsible for this suppressed stress responsiveness. However, it has subsequently been shown that suckling is a potent stimulator of the HPA axis and probably accounts for the tonically elevated trough plasma ACTH and corticosterone levels that occur during lactation (Walker *et al.*, 1992). Circulating levels of corticosterone are high in both late pregnancy and during lactation and may be responsible for the dampened responsiveness of the HPA axis during stress. One report suggested that the lactating rat was more sensitive to feedback (Schlein *et al.*, 1974), however, in this study dexamethasone was given and corticosterone, not ACTH, was measured as the experimental end point. A more recent report by Walker *et al* (1992) found no differences in the sensitivity to glucocorticoid feedback between lactating and virgin rats. Studies looking at feedback sensitivity during pregnancy have found either no difference or a decreased sensitivity (Keller-Wood, 1996; Owens *et al.*, 1987; see Chapter 5).

A recent study by da Costa *et al* (1996) examined the expression of the immediate early gene c-*fos* mRNA in specific brain areas of both late pregnant and lactating rats following restraint stress. The mRNA expression of c-*fos* in the PVN, the medial amygdala and the lateral septum, both of which possess connections with the PVN (Sawchenko & Swanson, 1983) was significantly less in both late pregnant and lactating rats following stress. Since c-*fos* mRNA expression is widely recognised as a marker of neuronal activation (Sagar *et al.*, 1988) these results suggest that the modulation of afferent inputs to the PVN may be involved in reducing the responsiveness of the HPA axis during both pregnancy and lactation. However, as mentioned previously, quantitative differences appear to exist between the stress responsiveness of the late pregnant and lactating rat. The latter has been shown not to respond to a physical stress (Lightman & Young III, 1989), although

others have shown a response to ether stress (Walker *et al.*, 1992). Thus, other mechanisms probably downstream from the afferent pathways may also have a role in attenuating the stress response.

Therefore, we went on to investigate whether changes in the forward drive, or in the negative glucocorticoid feedback signal on the HPA axis, or a combination of both are responsible for this attenuation of the neuroendocrine stress responses in pregnancy.

Why have an attenuated HPA axis response to acute stress during pregnancy? Epidemiological studies in several different human populations have demonstrated that indicators of an adverse intrauterine enviroment, including low birth weight are associated with increased incidence of cardiovascular disease (Barker et al., 1993), hypertension (Barker *et al.*, 1990) and non-insulin-dependent diabetes mellitus (Hales *et al.*, 1991) in adulthhood, supporting the hypothesis that early life events programme later blood pressure regulation and glucose homeostasis. Mechanisms underlying prenatal programming are unclear, although maternal malnutrition has been proposed to have a strong influence (Barker *et al.*, 1993; Hales *et al.*, 1991). An alternative mechanism is exposure of the fetus to maternal glucocorticoids. Exposure to excess glucocorticoids *in utero* results in retarded fetal growth (Reinisch *et al.*, 1978). More recently, it has been shown that adult offspring of pregnant rats treated with a moderate dose of dexamethasone (100 μg/kg/day) have elevated systolic blood pressures (Benediktsson *et al.*, 1993). This supports the hypothesis that prenatal exposure to glucocorticoids leads to the development of diseases, such as hypertension, in adult life (Edwards *et al.*, 1993). Under normal circumstances the fetus is protected from high maternal levels of physiological glucocorticoids by placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which catalyses the rapid conversion of active glucocorticoids (cortisol in humans and corticosterone in rodents) to inert 11-keto derivatives (cortisone and 11-dehydrocorticosterone, respectively). It is highly expressed in the placenta, particularly in the syncytiotrophoblast (Brown *et al.*, 1996), and maintains the low fetal glucocorticoid levels (Edwards *et al.*, 1993). Several *in vivo* studies have shown the importance of this enzyme in regulating the access of glucocorticoids to the fetus. When

carbenoxolone, a potent inhibitor of 11β-HSD2, is administered to the mother the adult offspring exhibit higher fasting plasma glucose levels (Lindsay *et al.*, 1996a) and elevated blood pressure (Lindsay *et al.*, 1996b). A recent *in situ* hybridisation study examined the expression of the 11β-HSD2 gene in the murine fetoplacental unit and found that from embryonic day 16.5 its mRNA expression in the placenta was switched off (Brown *et al.*, 1996). This result suggests that near term the 11β-HSD2 barrier to glucocorticoids is removed. However, this finding does not correlate with 11β-HSD activity data, which is present in rodent term placenta (Brown *et al.*, 1993; Burton & Turnell, 1968) and thus possibily reflects persisting 11β-HSD2 protein and/or the presence of the 11β-HSD1 isoform or even an unique isoform. Although the type 2 isoform is the predominant form in the placenta 11β-HSD1 activity is also present. Burton & Waddell, (1994) measured the *in vitro* activity of 11β-HSD1 in placental fragments isolated from day 16, day 19 and day 22 pregnant rats. 11β-reductase activity declined while 11β-dehydrogenase activity increased with advancing pregnancy, thus, there was a net increase in placental 11β-dehydrogenase activity towards term, consistent with a reduction in the transfer of active glucocorticoids between mother and fetus. However, at midterm 11β-HSD enzyme activity in the human, baboon and rat placenta appear to be predominantly reductive (Burton & Waddell, 1994). It is likely that the transfer of some maternal glucocorticoids to the fetus is important during early development since in the majority of species the fetus is incapable of synthesising its own glucocorticoids until relatively late in pregancy (Dupont *et al.*, 1991). In the rat, it has been reported that the fetal adrenal cortex develops the capacity to synthesise corticosterone only after day 17 of gestation (Milkovic *et al.*, 1973); however, a more recent report indicated that the fetus possessed this ability by day 14 of gestation (Cohen *et al.*, 1990). Thus placental 11β-HSD activity appears to regulate rather than exclude maternal glucocorticoids during fetal development.

The suppression of the HPA axis response to stress may be an additional adaptive response by the mother to protect the fetus from exposure to excess glucocorticoids. Free circulating levels, regardless of the higher CBG levels, have

been shown to be elevated during pregnancy, especially toward term (Nolten & Rueckert, 1981). In response to a stress the rise in plasma corticosterone levels will be superimposed on an already high baseline. With progressing pregnancy these high stress-induced levels may potentially swamp the protective 11β-HSD placental barrier and gain access to the fetal compartment at a time when excessive exposure will be deleterious to the fetus. It is of interest that the suppression of the maternal stress responsiveness by day 15 of pregnancy (Neumann *et al.*, 1998) coincides with the ability of the fetus to synthesise its own glucocorticoids (Cohen *et al.*, 1990).

Alternatively, or in addition, the attenuated HPA axis responsiveness may be a mechanism allowing the conservation of maternal energy stores which are required to support the developing fetus, particularly in late pregnancy (Knopp *et al.*, 1973; Metcalfe *et al.*, 1988), and during parturition. During stress the increased plasma corticosterone levels mobilise energy stores to allow the organism to overcome the stress. Thus, in the pregnant rat the lower stress-induced plasma corticosterone levels will cause less energy mobilisation and the conservation of these stores.

CHAPTER 4

Changes at the level of the PVN and in the responsiveness of the anterior pituitary corticotrophs during pregnancy

4.1 Introduction

The observed attenuation of HPA axis responses to acute stress in pregnancy (Neumann *et al.*, 1998; see Chapter 3) may reflect adaptations occurring in the central neural forward drive to the axis. The hypothalamic paraventricular nucleus (PVN) is the central coordinator of the stress response as it receives extensive afferent innervation from numerous brain regions (Cullinan *et al.*, 1993; Cunningham & Sawchenko, 1988; Gray *et al.*, 1989; Sawchenko & Swanson, 1982, 1983; Weller & Smith., 1982) through which inputs, both inhibitory and stimulatory, modulate the activity of the axis. Major inputs arise from catecholaminergic cell groups in the brainstem (Cunningham & Sawchenko, 1988; Sawchenko & Swanson, 1982) and from the bed nucleus of the stria terminalis (BNST) which relays inputs from several limbic structures including the hippocampus and amygdaloid complex (Cullinan *et al.*, 1993).

The parvocellular CRF neurones of the PVN provide the central neural drive to the pituitary-adrenocortical axis during the diurnal peak and in response to stressful stimuli (Akana & Dallman, 1992; Akana *et al.*, 1992). These neurones receive abundant innervation from both the noradrenergic A2 cell group (nucleus of the solitary tract, NTS) and several of the adrenergic C1, C2 and C3 cell groups in the brainstem (Cunningham *et al.*, 1990) and at the ultrastructural level both noradrenergic and adrenergic nerve terminals have been described on the dendrities and cell bodies of the CRF-containing neurones (Liposits *et al.*, 1986). Data from animal and human studies indicate that the brainstem noradrenergic system is activated alongside the HPA axis during stress (for review see Chrousos *et al.*, 1988) and catecholamines appear to stimulate the HPA axis through the release of CRF (Plotsky *et al.*, 1989).

A number of studies have investigated the regulation of gene expression and the release pattern into the HPB of several ACTH secretagogues including CRF. Regulation appears to occur in a stress specific manner with physical and psychological stressors exhibiting a different pattern of expression indicating that different afferent pathways and neurotransmitters may be involved in these stresses (Harbuz & Lightman, 1989). Following acute stresses such as restraint or swim stress or i.p. hypertonic saline the levels of parvocellular CRF and AVP but not oxytocin (OXT) mRNAs increased (Harbuz & Lightman, 1989; Herman & Sherman, 1993; Kalin et al., 1994) and these changes are reflected in studies examining hypothalamic content (Lightman & Young III, 1988; Moldow et al., 1987). Intracisternal injection of colchicine disrupts fast axonal transport of secretatogues from the parvocellular cell bodies in the PVN, thus creating an isolated pool of secretagogues in the median eminence. Decreases in this pool following stimulation correlate with a release of secretagogues into the HPB; it was found that exposure to a psychological stressor decreased immunoreactive OXT (irOXT) but not the irCRF content of the median eminence (Romero et al., 1993). This indicates that in addition to driving the ACTH response during severe stress CRF functions in a permissive role in response to milder stressors, by allowing weaker secretagogues such as AVP and OXT to exert their effects (Plotsky *et al.*, 1985a).

The removal of the glucocorticoid negative feedback signal by adrenalectomy (adx) is a powerful stimulus to the HPA axis (Plotsky & Sawchenko, 1987). Several hybridisation studies have investigated the effects on the expression of the CRF and AVP genes. Pharmacological adx achieved with metyrapone, an 11β-hydroxylase inhibitor, which prevents the conversion of 11-deoxycorticosterone to corticosterone, caused increased immunohistochemical staining for CRF and AVP in the PVN, median eminence CRF and AVP contents and concentrations in HPB by 72 h (Plotsky & Sawchenko, 1987). With an intronic probe to the CRF gene it has been demonstrated that within 30 min of metyrapone the CRF primary transcript levels are increased (Herman *et al.*, 1992). Other hybridisation studies following surgical adx found an increase in CRF mRNA in the PVN ranging from 60% to 275% over baseline levels (Beyer *et al.*, 1988; Imaki *et al.*, 1991; Young *et al.*, 1986). However,

other reports showed more modest increases in CRF mRNA levels (Albeck *et al.*, 1994; Swanson & Simmons, 1989). Quantitative differences between studies are likely to reflect different methods of quantification. In contrast, AVP mRNA expression and immunoreactivity in the parvocellular PVN consistently increase following adx (Albeck *et al.*, 1994; Davis *et al.*, 1986; Sawchenko, 1987) and these findings confirm *in vitro* data where the ratio of AVP:CRF released from the median eminence increased from 2:1 to 9:1 following adx (Holmes *et al.*, 1986).

A recent report using *in situ* hybridisation examined the basal expression of CRF mRNA in the PVN during pregnancy and found that parvocellular CRF mRNA expression on day 21 of pregnancy was significantly decreased (Douglas & Russell, 1994), indicating either an increased inhibitory influence consistent with an increased negative feedback signal or decreased excitatory input at the level of the parvocellular cell bodies. We sought to confirm this observation with a different technique. We used a cRNA (riboprobe) rather than an oliogonucleotide probe because riboprobes possess a higher specific activity therefore producing a stronger signal upon hybridisation and making it more sensitive for measuring small changes in mRNA expression (Tecott *et al*, 1987).

CRF exerts its physiological effects by binding to G-protein-linked membrane-bound receptors (Chang *et al.*, 1993) and *in vitro,* CRF increases intracellular cAMP levels in cultured anterior pituitary cells (Aguilera *et al.*, 1986). The CRF receptors characterised so far are encoded by two separate genes and differ in their anatomical distribution and pharmacological profiles. The $CRFR_1$, which was the first to be isolated, has a widespread central distribution (Wong *et al.*, 1994) and is the dominant form in the pituitary (Potter *et al.*, 1994). CRF has a higher affinity for this receptor subtype than either the related mammalian urocortin, amphibian sauvagine or fish urotensin I. The $CRFR_2$ exists as two splice variants, $CRFR_{2\alpha}$ and $CRFR_{2\beta}$, and these have distinct tissue distributions. $CRFR_{2\alpha}$ is expressed in limited areas of the brain, including the lateral septum and ventromedial hypothalamus, whereas $CRFR_{2\beta}$ is found in peripheral tissues including the heart and skeletal muscle as well as in the brain, where it is associated with blood vessels (Lovenberg *et al.*, 1995a). Urocortin, sauvagine and urotensin I are all more potent then CRF at increasing

cAMP in cells transfected with either $CRFR_2$ subtype (Kishimoto *et al.*, 1995; Perrin *et al.*, 1995, Vaughan et al., 1995).

Regulation of pituitary CRFR binding has been readily demonstrated in rats given exogenous CRF or glucocorticoid treatment and after exposure to stress or following adx (Hauger & Aguilera, 1993; Hauger *et al.*, 1987,1990; Wynn *et al.*, 1985). Later studies examining the regulation of the $CRFR_1$ gene found comparable results. In cultured anterior pituitary cells CRF dose- and time-dependently decreased CRF receptor mRNA expression (Pozzoli *et al.*, 1996; Sakai *et al.*, 1996) as did *in vitro* and *in vivo* glucocorticoid treatment, exposure to either acute or chronic immobilisation stress and adx (Makino *et al.*, 1995; Sakai *at al.*, 1996).

Neumann *et al*, (1998) reported that the ACTH response to exogenous CRF *in vivo* was significantly attenuated from day 15 of pregnancy, indicating that an adaptation may have occurred at the level of the anterior pituitary resulting in decreased sensitivity to CRF.

We used receptor autoradiography to examine whether there was an alteration in the density of CRF binding sites in the anterior pituitary during pregnancy. In addition we examined the accumulation of cAMP in pituitary segments and acutely dispersed anterior pituitary cells isolated from pregnant and virgin rats, in response to exogenous CRF.

4.2 Material and Methods

4.2.1 CRF and AVP mRNA *in situ* hybridisation histochemistry

For details see Chapter 2. Briefly, individually housed day 10, 16 & 21 pregnant and virgin rats were tranferred separately to the experimental room where they were decapitated between 09.30 h and 10.30 h to minimize the effects of stress. The brains were rapidly removed and immediately frozen on dry ice. 20 μm cryostat-cut coronal brain sections mounted onto gelatin and poly-L-lysine coated slides were fixed in 4% paraformaldehyde, incubated with prehybridisation buffer for 2 h at 50 °C and then hybridised overnight at 50 °C with ^{35}S-labelled antisense cRNA probes.

T3 polymerase was used to transcribe the CRF riboprobe from a Xba I-linearised pBluescript plasmid containing the rat CRF cDNA insert and SP6 polymerase was used to transcribe the AVP riboprobe from a ECoR I-linearised pGEM3 plasmid containing the rat AVP cDNA insert.

The following morning the sections were washed in reducing salt concentration to a maximum of 0.1 x SSC at 60 °C for 60 min. Then the sections were dehydrated with increasing concentrations of ethanol in 0.3 M ammonium acetate and allowed to air-dry. The sections were then placed against film for 7 days to check that the hybridisation signal was specific. Afterwards they were dipped in photographic emulsion and stored at 4 °C for 21 days before being developed and counterstained with pyronin Y. The length of time that the sections were exposed against film and after dipping in autoradiographic emulsion were based on previous trials in the laboratory.

To determine the specificity of the hybridisation signal adjacent slides were hybridised with ^{35}S-labelled sense cRNA probes which were not complementary to the CRF or AVP mRNAs. They were both transcribed using T7 polymerase. For details see Chapter 2.

Analysis was carried out using a high power microscope (objective x40) linked to a computer-based image analysis system (See scan). See Chapter 2. The number of silver grains per neurone in the medial parvocellular PVN were determined; for each animal, measurements were made on three sections. Background measurements were made on adjacent areas of tissue and automatically subtracted from the PVN measurements. The group means were then calculated and the data expressed as percentages of virgin control.

4.2.2 [^{125}I]CRF binding in the pituitary

For details see Chapter 2. Briefly, day 10, 16 & 21 pregnant and virgin rats were decapitated between 9.30 h and 10.30 h and the pituitary glands removed and immediately frozen on dry ice. 20 μm cryostat-cut horizontal pituitary gland sections were mounted onto gelatin and poly-L-lysine-coated slides and desiccated overnight at 4 °C.

For receptor autoradiography, sections were incubated with 0.2 nM [^{125}I]ovineCRF (oCRF) (specific activity 2200 Ci/mmol) at room temperature for 60 min. Non-specific binding was determined in the presence of unlabelled oCRF (10-6 M). Sections were then washed three times in BSA-free Tris-HCl buffer at 4 °C for 2 min each, dried in a stream of cold air and exposed to film for 22 days.

Analysis of the autoradiographs was performed using a microscope (objective x10; additional magnification x1.6) linked to a computer-assisted image analysis system (Joyce-Loebl Micromagiscan). Silver grain area per unit area (silver grain density), was measured within a 100 μm^2 frame over each section. Total binding was measured over two different areas of the anterior pituitary, one area of the intermediate lobe and one area of the posterior lobe per section; for each animal measurements were made on three sections. Background measurements were made on adjacent areas off the tissue for each pituitary and the background silver grain density subtracted from the tissue grain density. For non-specific binding, three measurements of pituitary grain density were made on sections incubated with excess unlabelled oCRF; the pituitary lobes were not distinguished.

Mean silver grain density values were calculated for each rat per pituitary lobe and the mean non-specific binding values subtracted from the total. Group means were then calculated (Fig 4.3a).

4.2.3 *In vitro* CRF-induced anterior pituitary cAMP levels

4.2.3.1 Anterior pituitary segments

For details see Chapter 2. Briefly, day 10, 16 & 20 pregnant and virgin rats were transferred singly to the experimental room between 9.30 h and 10.30 h where they were decapitated and the anterior pituitaries rapidly removed. Each anterior pituitary was cut into eight equally-sized segments under a dissecting microscope by hand with a scapel blade. The segments were then incubated with DMEM and 0.5 mM IBMX, a phosphodiesterase inhibitor for 15 min at 37 °C in 24 well cluster plates. The segments were then challenged with either 20 nM human CRF (hCRF)

(final concentration 10 nM) or DMEM (vehicle) for 10 min and the reaction stopped with the addition of ice-cold 0.2 N HCl.

Total cAMP levels were measured using a specific and sensitive radioimmunoassay (Dufau et al., 1973). See Chapter 2 for details.

4.2.3.2 Acutely dispersed anterior pituitary cells

For details see Chapter 2. Briefly, day 21 pregnant and virgin rats were decapitated between 9.30 h and 10.30 h and the anterior pituitaries rapidly removed. Each anterior pituitary was finely chopped by hand with a scalpel blade and pooled into their respective groups; pregnant and virgin. The cells were dispersed with trypsin at 37 ^{o}C and after approximately 25 min the trypsin was inactivated by the addition of a trypsin inhibitor. The cells (2 x 10^5 cells/tube) were then challenged with hCRF; 0.1, 1 or 10 nM or DMEM for 10 min at 37 °C. The reaction was initiated with the addition of the cells and terminated with the addition of ice-cold 0.2 N HCl.

Total cAMP levels were determined using a specific highly sensitive acetylated cAMP radioimmunoassay. See Chapter 2 for details.

4.3 Results

4.3.1 CRF and AVP mRNAs: basal expression

In both *in situ* hybridisation studies the hybridisation signal was primarily located in the cytoplasm of the cells and hybridisation with the sense probes in adjacent sections did not produce a signal (Fig 4.1). The analysis of the *in situ* hybridisation for AVP mRNA was carried out in the dorsomedial region of the PVN since this area contains the highest density of AVP-positive CRF neurones (Whitnall & Gainer, 1989). We also detected a few scattered magnocellular neurones within this region which expressed extremely high levels of AVP mRNA within this region, however, these were not included in the analysis.

The number of silver grains/neurone, corresponding to CRF mRNA expression, in the medial parvocellular region of the PVN decreased during pregnancy (Fig

4.1b). On days 10 and 16 of pregnancy, the CRF mRNA expression was slightly decreased, although not significantly, compared to the virgin controls. On day 21 of pregnancy, however, the number of silver grains/neurone was significantly decreased ($p < 0.05$ vs. virgin) by approximately 30% compared to virgin controls.

The silver grain density over dorsomedial PVN neurones, representing AVP mRNA expression, did not significantly differ between virgin and any of the days of pregnancy examined (Fig 4.2).

4.3.2 [^{125}I]oCRF autoradiography

This study was a collaboration with Prof. R Landgraf's group in the Neuroendocrinology laboratories at the Max Planck Institute of Psychiatry, Munich, Germany.

The silver grain density, representing specific [^{125}I]oCRF binding site density, was significantly reduced in the anterior pituitary on all days of pregnancy examined ($p < 0.01$ vs. virgin) (Fig 4.3b). On day 10 and 16 of pregnancy the specific [^{125}I]CRF binding was decreased by approximately 50%. There was a further significant decrease of 20% on day 21 of pregnancy ($p < 0.05$ vs. day 10 & 16 of pregnancy).

Specific [^{125}I]CRF binding in the intermediate lobe was significantly reduced ($p < 0.05$ vs. virgin) on day 21 of pregnancy only (Fig 4.3c).

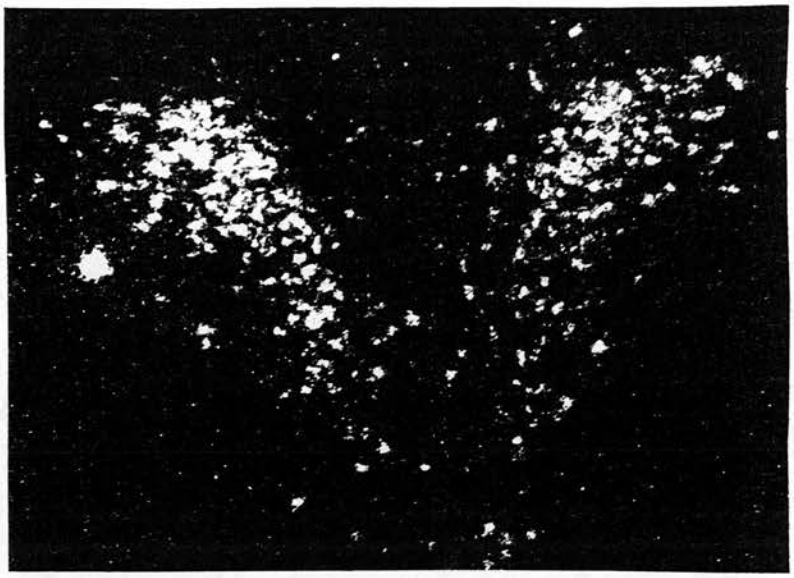
The binding of [^{125}I]CRF in the posterior pituitary was undetectable above the non-specific binding values.

4.3.3 *In vitro* CRF-induced anterior pituitary cAMP levels in virgin and pregnant rats

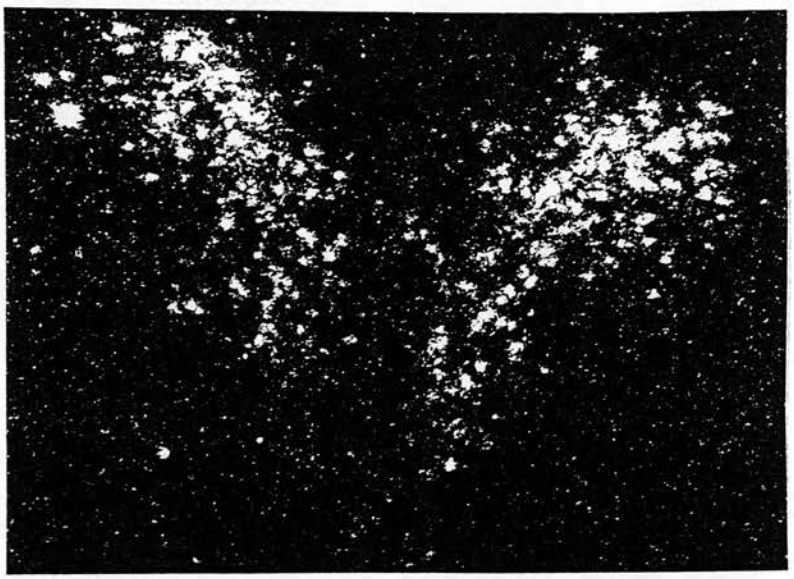
4.3.3.1 Pituitary segments

In the presence of the phosphodiesterase inhibitor, IBMX, the basal and the CRF-stimulated cAMP accumulation were significantly lower in pituitary segments isolated from day 16 & 20 pregnant rats compared to the respective virgin controls ($p < 0.05$ vs. virgin group, basal and CRF-stimulated cAMP values on day 16 of pregnancy; basal values on day 20 of pregnancy; and $p < 0.01$ vs. virgin group, CRF-

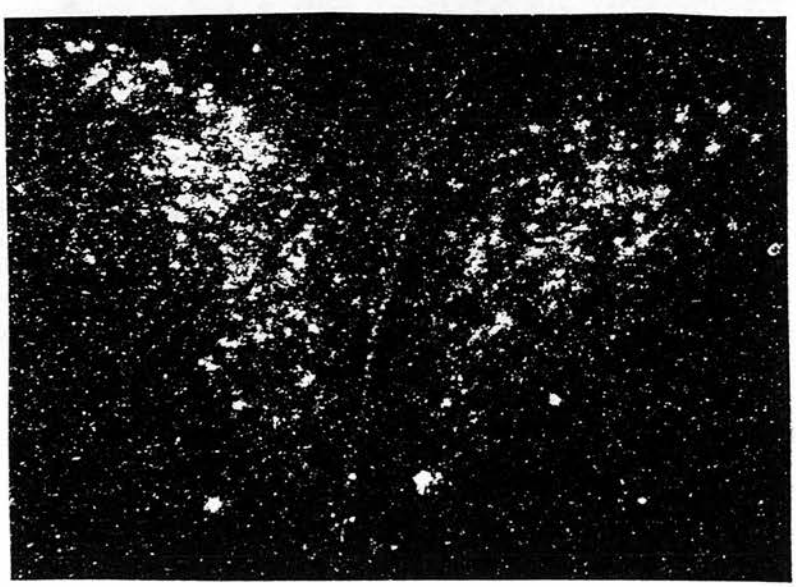
A



B



C



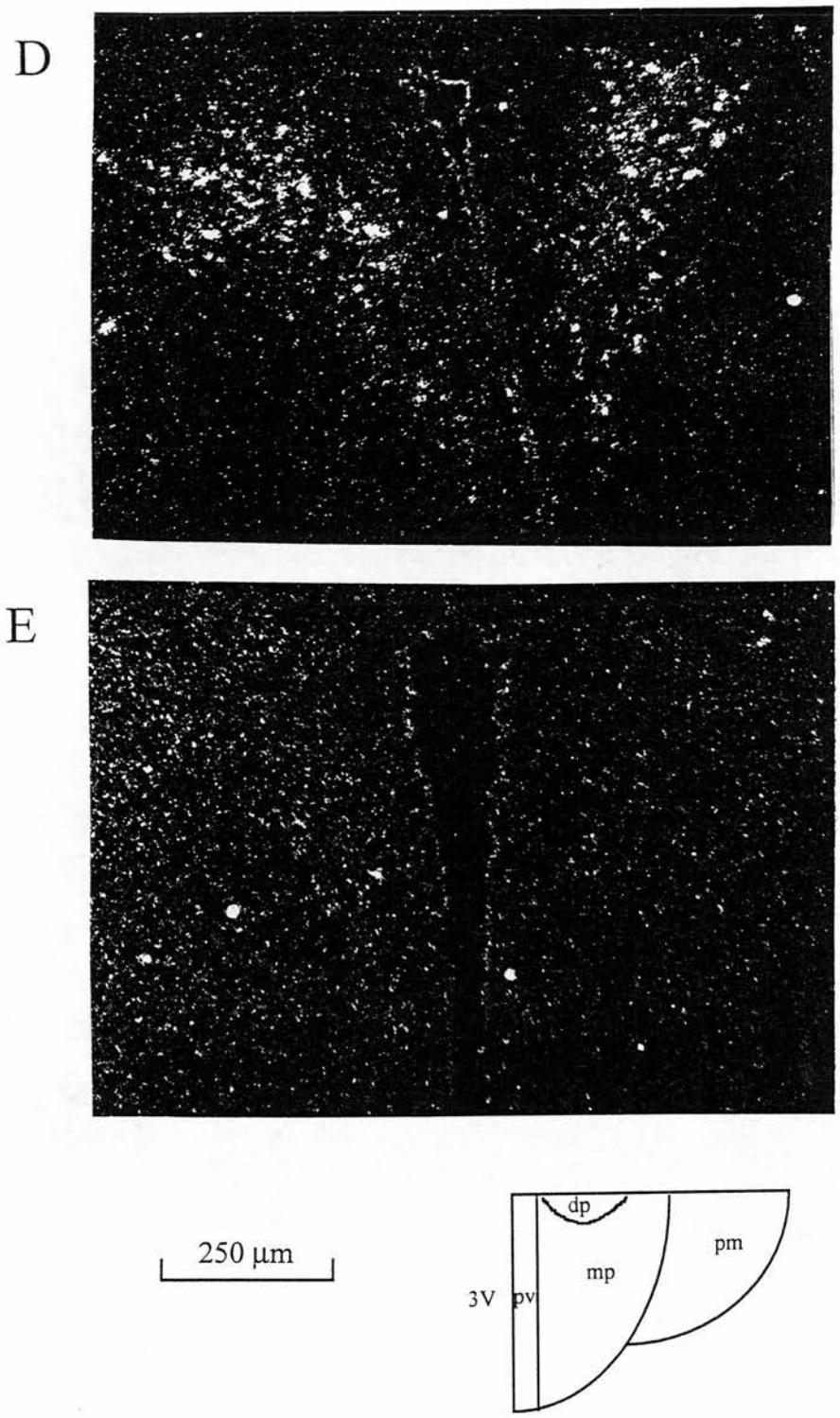


Fig 4.1a: Photographs of CRF mRNA hybridisation in the PVN of **A** virgin, **B** day 10 pregnant, **C** day 16 pregnant & **D** day 21 pregnant rats. **E** sense probe hybridisation. Regions of the PVN: pm = posterior magnocellular ; mp = medial parvocellular, dp = dorsal parvocellular ; & pv = periventricular. 3V = third ventricle.

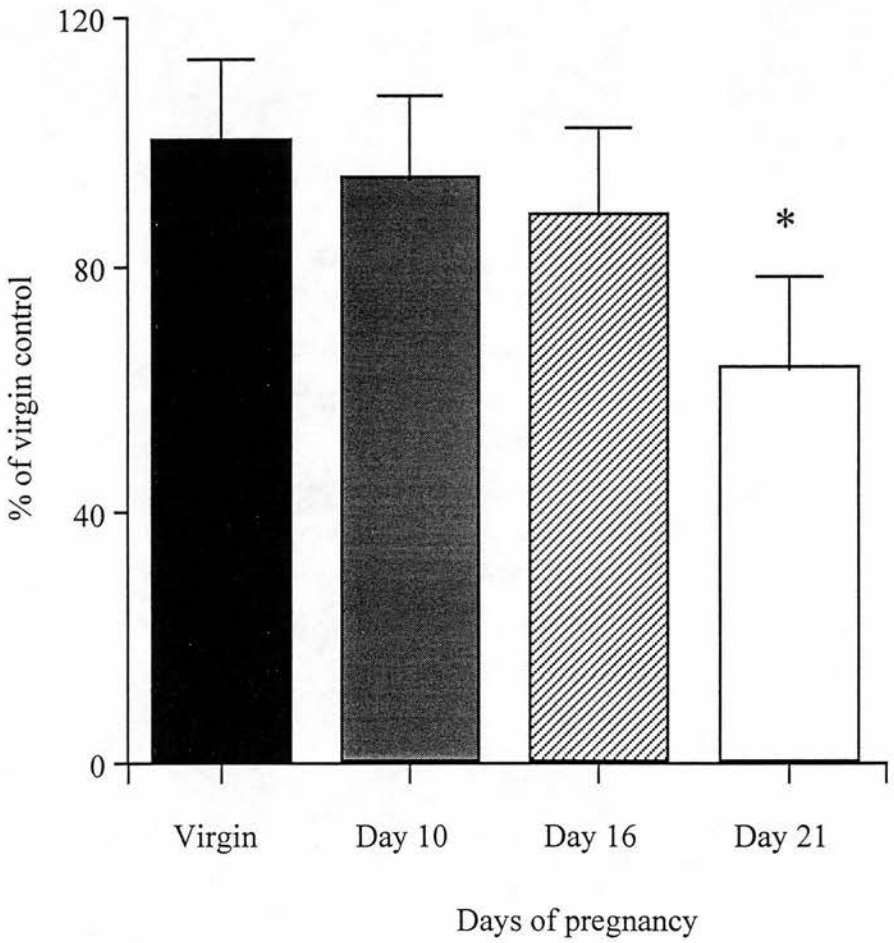


Fig 4.1b: CRF mRNA expression in the medial parvocellular region of the PVN. Sections were analysed by a computer-based image analysis system (SeeScan) as the mean number of silver grains/neurone and data expressed as % of virgin control ± S.E.M. ANOVA on raw data followed by Newman-Keuls student's test: * $p < 0.05$ vs. virgin group, n = 6/group.

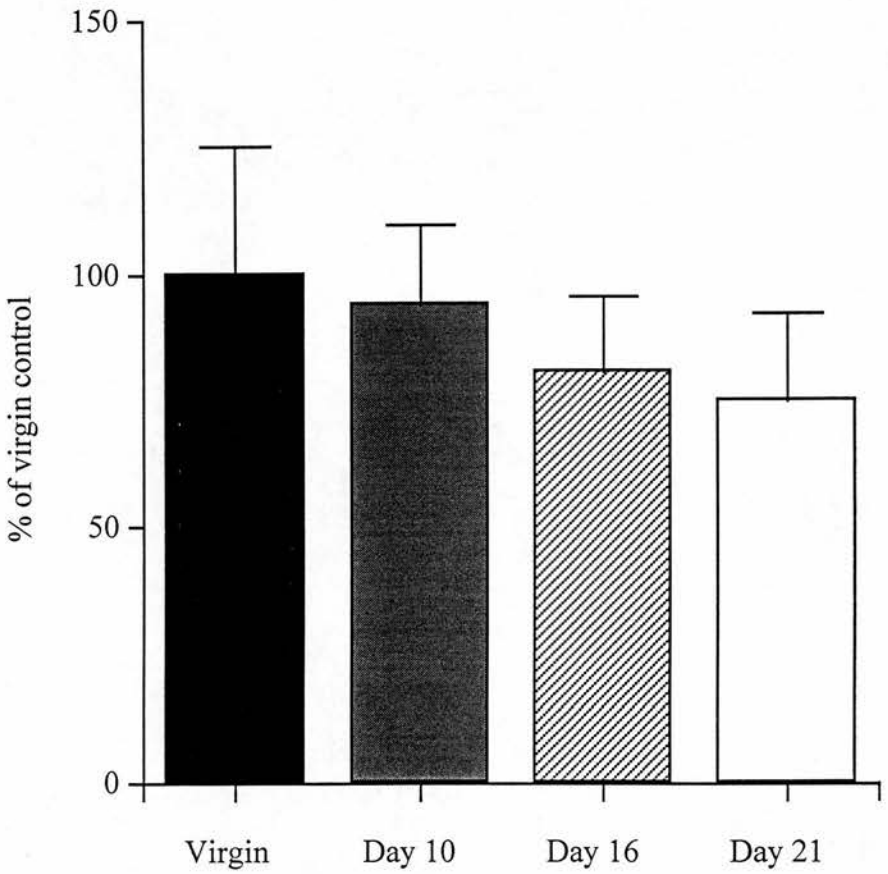
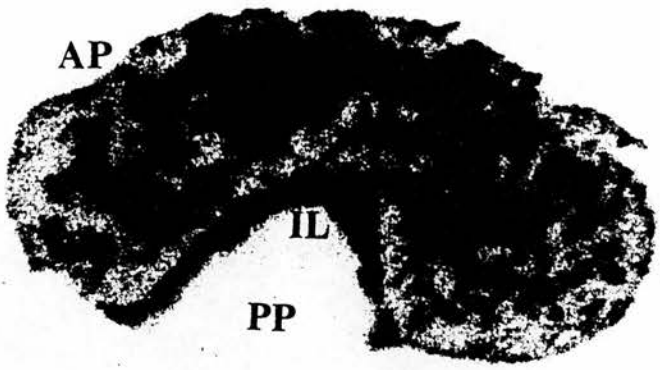
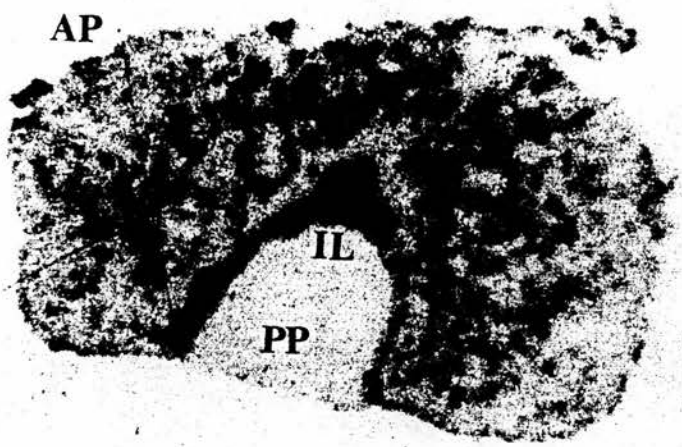


Fig 4.2: AVP mRNA expression in the dorsal medial parvocellular region of the PVN. Sections were analysed by a computer-based image analysis system (SeeScan) as the mean number of silver grains/neurone and data expressed as % of virgin control ± S. E.M.. ANOVA: no sigficance (n.s) between groups, n = 6/group.

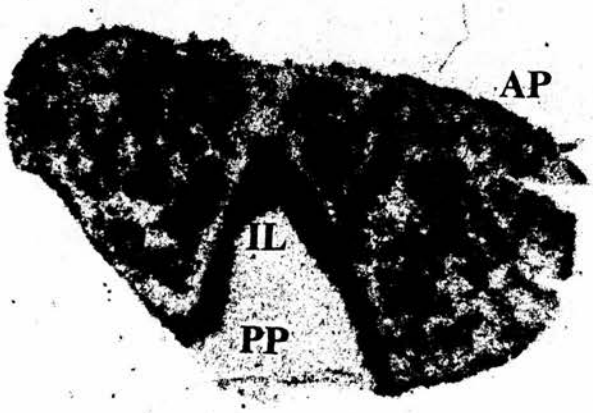
A



B



C



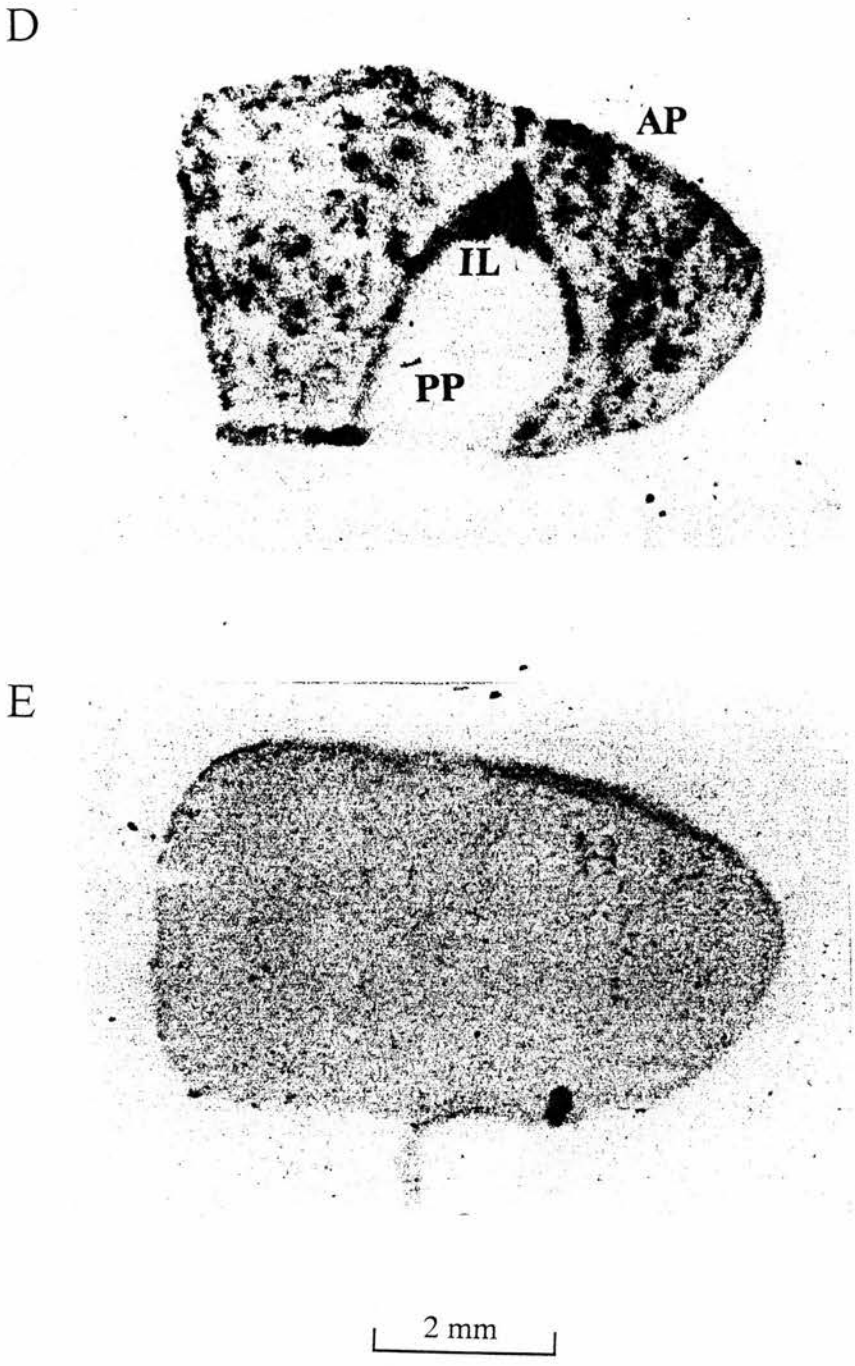


Fig 4.3a: Representative photographs of the autoradiographs of [^{125}I]CRF binding in whole pituitary sections. **A** - virgin, **B** - day 10 of pregnancy, C - day 16 of pregnancy, **D** - day 21 of pregnancy & **E** - non-specific binding. **AP** - anterior pituitary, **IL** - intermediate lobe & **PP** - posterior pituitary.

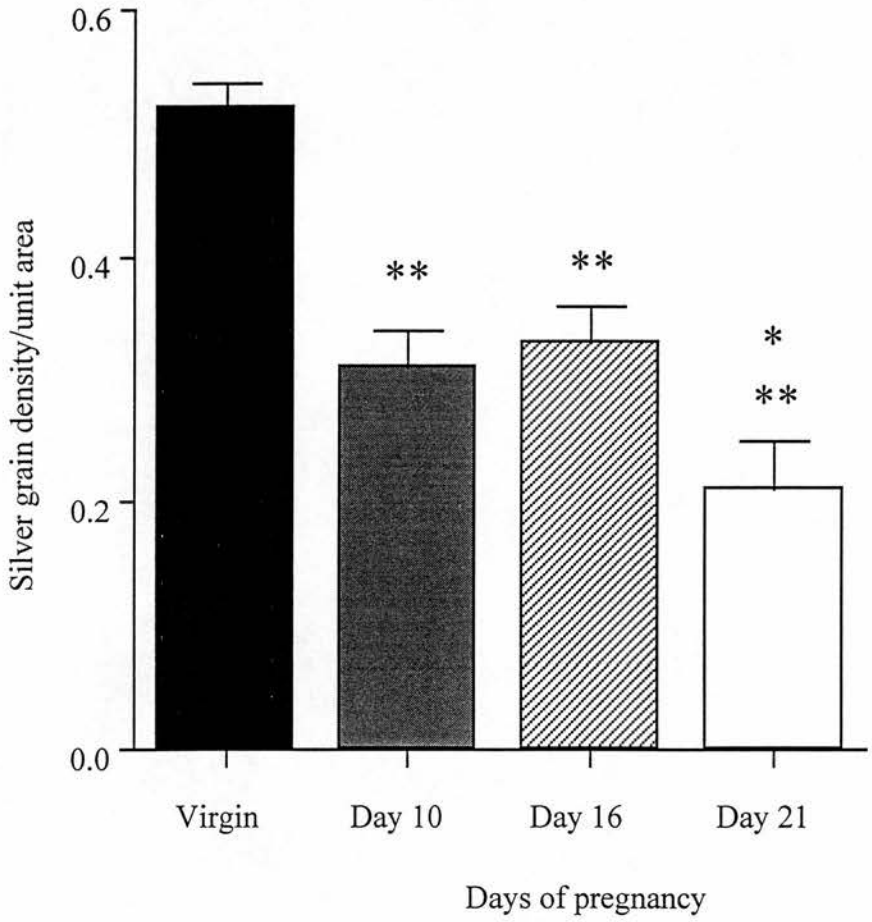


Fig 4.3b: Specific binding of [^{125}I]oCRF in the anterior lobe from pituitaries collected from day 10, 16 & 21 pregnant and virgin rats (n = 6/group). Film was analysed by a computer-based image analysis system (Joyce-Loebl Magiscan) and data expressed as mean ± S.E.M. silver grain density over a unit are. ANOVA followed by Newman-Keuls student's test: * $p < 0.05$ vs. day 10 and 16 of pregnancy and ** $p < 0.01$ vs. virgin group.

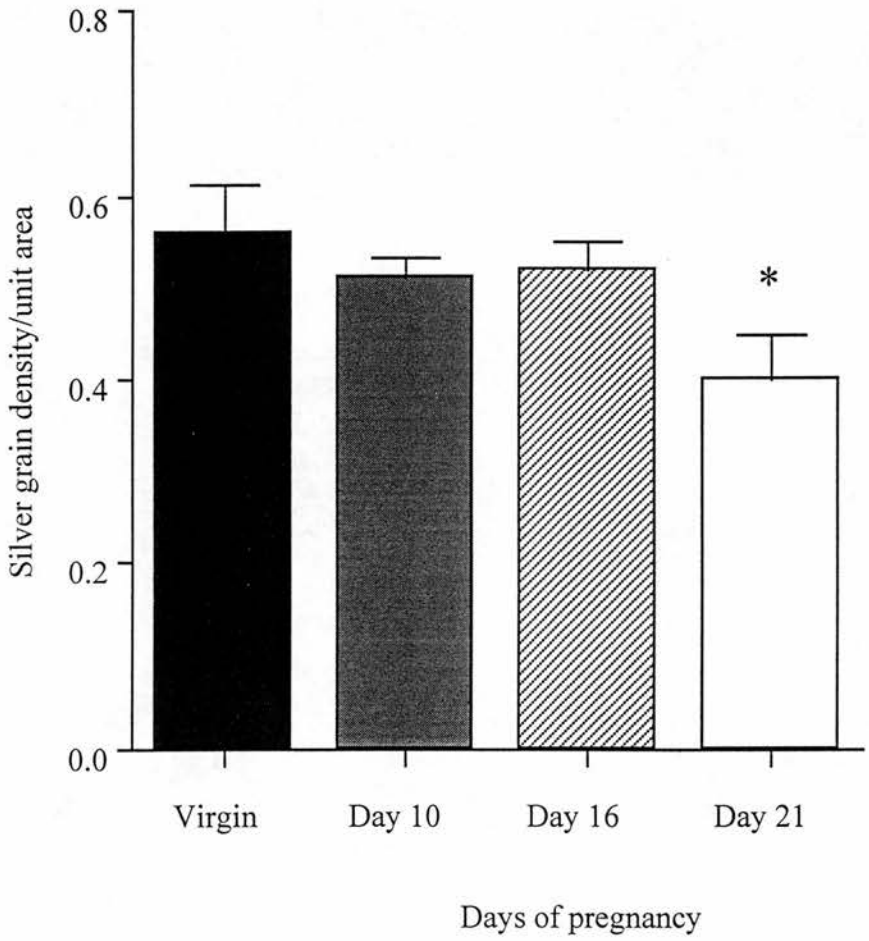


Fig 4.3c: Specific binding of [^{125}I]oCRF in the intermediate lobe of pituitaries collected from day 10, 16 & 21 pregnant and virgin rats (n = 6/group). Film was analysed by a computer-based image analysis system (Joyce-Loebl Magiscan) and data expressed as mean ± S.E.M. silver grain density over a unit area. ANOVA followed by Newman-Keuls student's test: *$p < 0.05$ vs. virgin group.

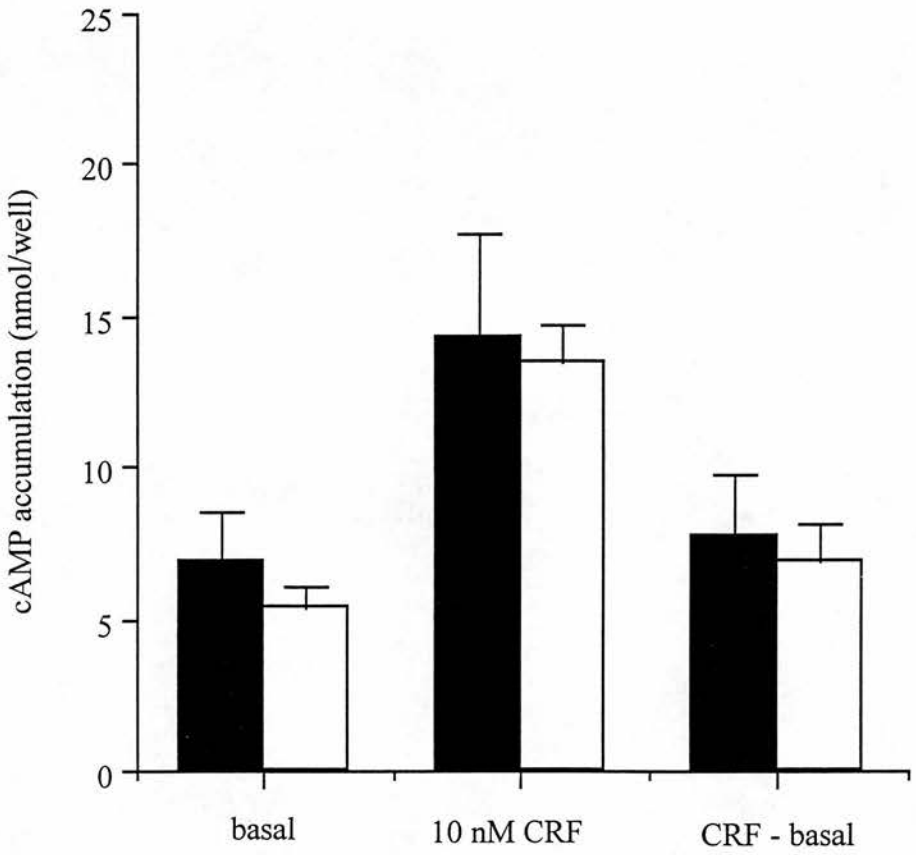


Fig 4.4a: cAMP accumulation in anterior pituitary segments isolated from (■) virgin and (□) day 10 pregnant rats, n = 4 and 6, respectively. Anterior pituitary segments were incubated *in vitro* with 10 nM hCRF or vehicle (DMEM) for 10 min and the reaction was stopped with the addition of ice-cold 0.2 N HCl. Data expressed as mean ± S.E.M. nmol cAMP accumulation/well.

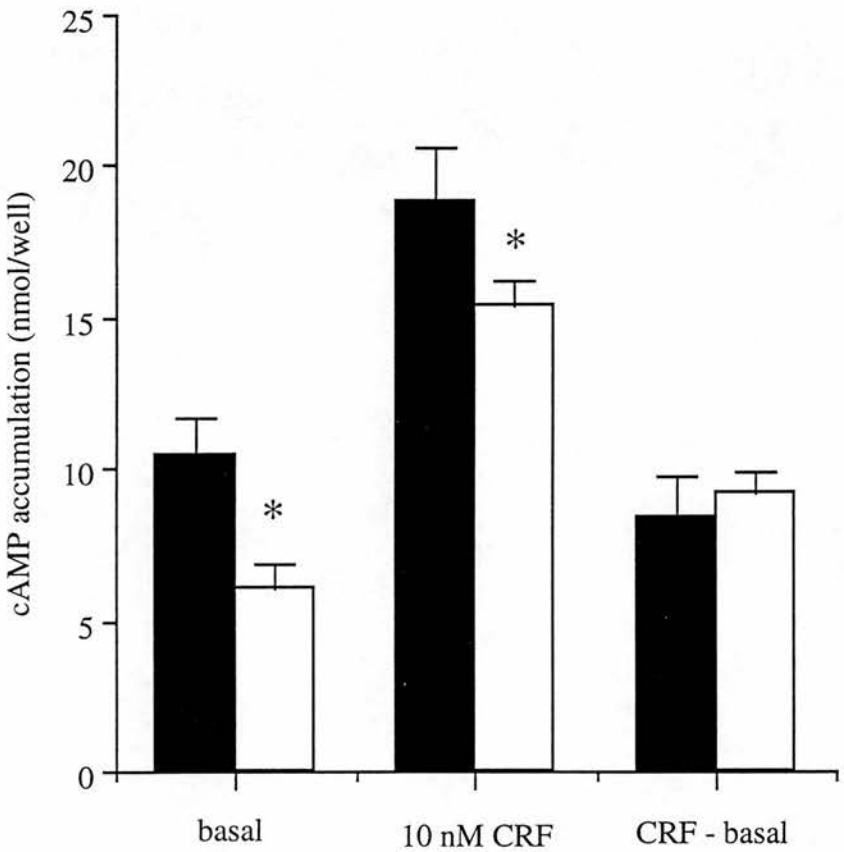


Fig 4.4b: cAMP accumulation in anterior pituitary segments isolated from (■) virgin and (□) day 16 pregnant rats, n = 4 and 6, respectively. Anterior pituitary segments were incubated *in vitro* with 10 nM hCRF or vehicle (DMEM) for 10 min and the reaction was stopped with the addition of ice-cold 0.2 N HCl. Data expressed as mean ± S.E.M. nmol cAMP accumulation/well. Students t test: * $p < 0.05$ vs. virgin group.

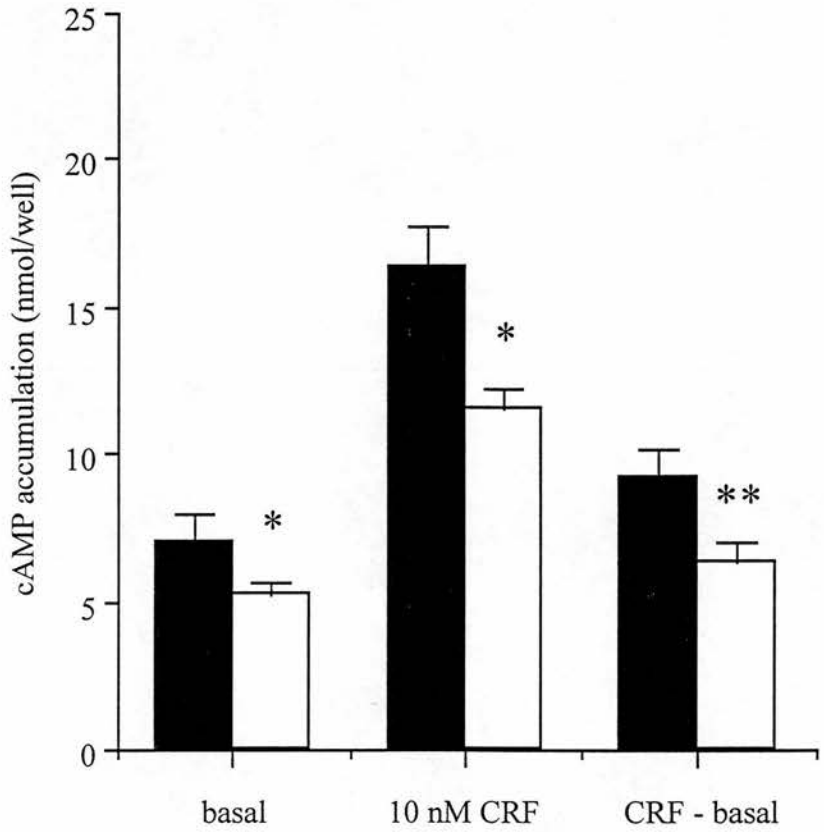


Fig 4.4c: cAMP accumulation in anterior pituitary segments isolated from (■) virgin and (□) day 20 pregnant rats, n = 8 in both groups. Anterior pituitary segments were incubated *in vitro* with 10 nM hCRF or vehicle (DMEM) for 10 min and the reaction was stopped with the addition of ice-cold 0.2 N HCl. Data expressed as mean ± S.E.M. nmol cAMP accumulation/well. Students t test: * $p < 0.05$, ** $p < 0.01$ vs. virgin group

stimulated value on day 20 of pregnancy) (Figs 4.4b & 4.4c). No difference was found in pituitary segments from day 10 of pregnancy compared to their virgin controls (Fig 4.5a).

Importantly, the CRF-induced increment (CRF-stimulated minus basal) in cAMP accumulation was significantly less on day 20 of pregnancy ($p < 0.05$ vs. virgin group) compared to their virgin controls (Fig 4.4c).

Anterior pituitary weights did not differ between virgin (11.14 ± 0.20 mg, n = 16), day 10 (11.27 ± 0.14 mg, n = 6), day 16 (11.60 ± 0.31 mg, n = 6) and day 20 pregnant rats (12.10 ± 0.35 mg, n = 8)).

4.3.3 Acutely dispersed anterior pituitary cells

In the absence of the phosphodiesterase inhibitor, IBMX, the basal cAMP accumulation was significantly greater in the cells from day 21 pregnant rats compared to the virgin group ($p < 0.05$ vs. virgin controls) (Fig 4.5a). In both pregnant and virgin groups the cAMP accumulation increased dose-dependently with hCRF. At a concentration of 1 nM hCRF, the cAMP levels in the cells isolated from day 21 pregnant rats were significantly lower compared to the virgin group ($p < 0.01$ vs. virgin controls). This effect was not seen at the higher dose of hCRF (10 nM).

The CRF-induced increments (CRF-stimulated minus basal) in cAMP levels in response to all three doses of hCRF used were significantly reduced in the cells isolated from the day 21 pregnant rats compared to the virgin group ($p < 0.01$ vs. virgin controls, 0.1 and 1 nM hCRF; $p < 0.05$ vs. virgin controls, 10 nM hCRF) (Fig 4.5b).

4.4 Discussion

4.4.1 CRF and AVP mRNAs: basal expression

We confirmed the previous finding, using a cRNA probe rather than an oliogonucleotide probe in the *in situ* hybridisation technique (Douglas & Russell, 1994) of a significant reduction in basal parvocellular CRF mRNA expression in the PVN on day 21 of pregnancy, with a trend towards decreased expression on day 10

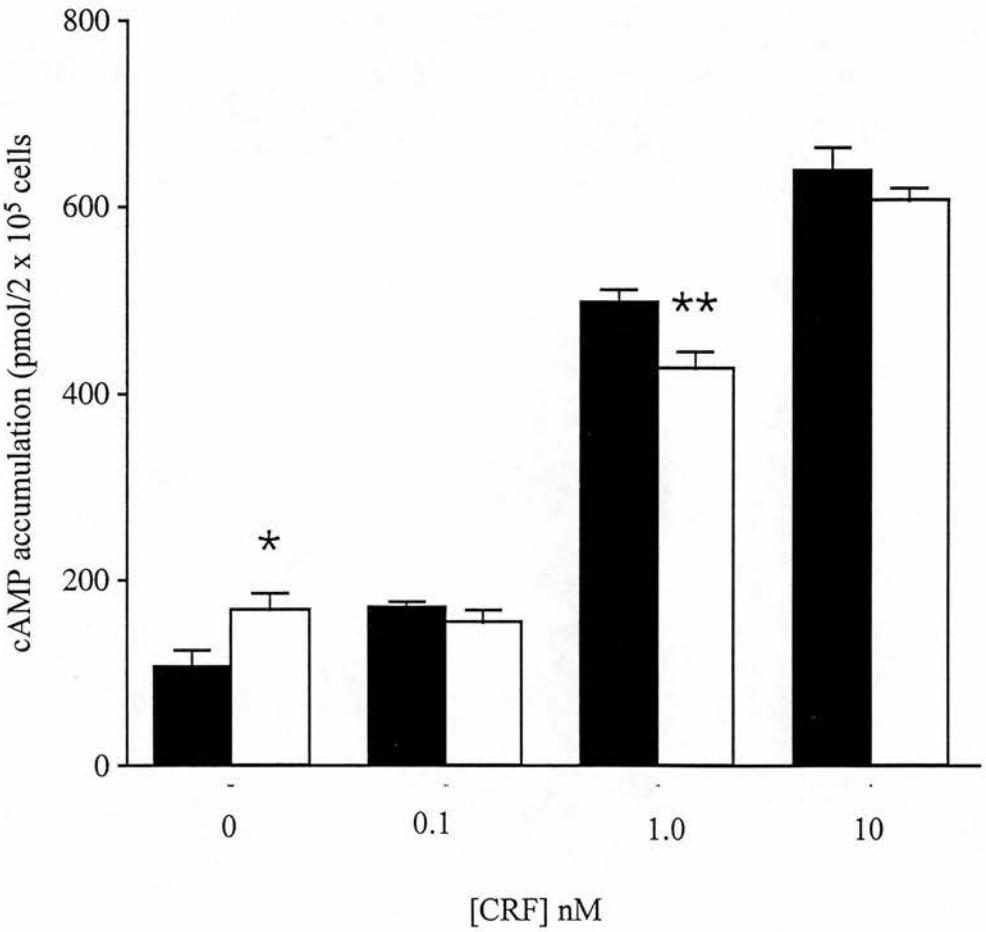


Fig 4.5a: cAMP accumulation in acutely dispersed anterior pituitary cells isolated from (■) virgin and (□) day 21 pregnant rats, n = 4 cell preparations/group from 2 virgin and pregnant rats in total. Cells were incubated *in vitro* with 0 (basal), 0.1, 1.0 & 10 nM hCRF for 10 min and the reaction was stopped with ice-cold 0.2 N HCl. Data expressed as mean ± S.E.M. pmol cAMP accumulation/2 x 10^5 cells. Student's t test * $p < 0.05$, ** $p < 0.01$ vs. virgin group.

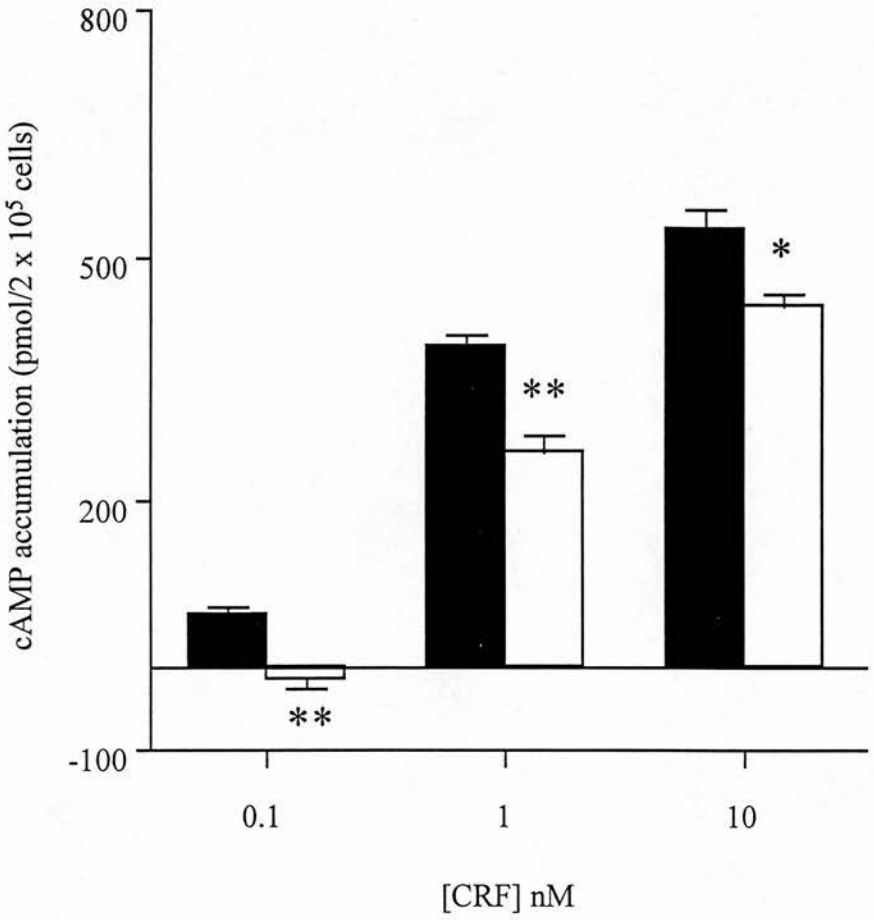


Fig 4.5b: cAMP accumulation in acutely dispersed anterior pituitary cells isolated from (■) virgin and (□) day 21 pregnant rats, n = 4 cell preparations/group from 2 virgin and pregnant rats in total. Cells were incubated *in vitro* with 0 (basal), 0.1, 1.0 & 10 nM hCRF for 10 min and the reaction was stopped with ice-cold 0.2 N HCl. Data expressed as mean ± S.E.M. pmol cAMP accumulation/2 x 10^5 cells. Student's t test * $p < 0.05$, ** $p < 0.01$ vs. virgin group.

and 16 of pregnancy. In addition to measuring CRF mRNA expression we also examined the basal mRNA expression of the other major ACTH secretagogue, AVP. Measurements were made in the dorsomedial parvocellular region of the PVN as this is the topographical location of the AVP-containing CRF neurones (Whitnall et al., 1989). The levels of parvocellular AVP mRNA expression did not change significantly with pregnancy although again there was a trend towards lower levels of expression as pregnancy progressed. However, caution is appropriate in interpreting this result as silver grains were localised only in a few scattered cells in the dorsomedial parvocellular region. For the analysis we employed a thresholding method where parvocellular neurones were detected as expressing AVP mRNA if the number of silver grains in the neurone was five times greater than background. Other studies have also employed a similar technique to analyse rapid changes in parvocellular AVP mRNA levels and found inconsistent results (Harbuz & Lightman, 1988; Ma *et al.*, 1997) with no change in the earlier study, but an increase in the later study in response to stress, indicating that the threshold method may not be sensitive enough to detect a decrease from an already low level of mRNA expression.

Parvocellular CRF neurones receive information from other brain regions and from the periphery via the brainstem. Major inputs arise from ascending projections from catecholaminergic cell groups in the brainstem. Several studies have shown that under basal conditions following unilateral transection of these pathways, with the specificity of the lesions confirmed histologically, there is a decrease in both the irCRF content of the parvocellular PVN and median eminence and mRNA expression in the paravocellular PVN. Thus, CRF expression appears to be under a tonic stimulatory influence from these ascending pathways (Kiss *et al.*, 1996; Sawchenko, 1988). Both noradrenergic and adrenergic synapses have been identified on the dendrites and cell bodies of CRF neurones (Liposits et al., 1985). Thus, the reduced CRF mRNA expression on day 21 of pregnancy may be a consequence of these pathways being inhibited. The inhibitory influence of endogenous opioid peptides (EOPs) on the hypothalamic magnocellular oxytocin cell bodies during pregnancy is well characterised (Hartman et al., 1986; Leng et al., 1988) and is

proposed to be mediated by μ-opioid receptors acting presynaptically on the noradrenergic A2 (NTS) brainstem afferents (Onaka et al., 1995; Russell et al., 1989). During pregnancy the hypothalamic content of β-endorphin has been shown to increase (Wardlaw & Frantz, 1983) and using an immunofluorescence double-labelling technique NTS afferents were found to innervate the parvocellular CRF neurones (Cunnungham & Sawchenko, 1988). Thus in pregnancy there may be an enhanced inhibitory β-endorphin tone on the nerve terminals of the NTS inputs to the CRF neurones thereby inhibiting CRF gene expression. However, although CRF mRNA after administration of naloxone was not examined, we have previously found it does not increase basal ACTH secretion in pregnancy (see Chapter 6) indirectly indicating that there is not an inhibitory opioid tone on the NTS afferents to the CRF neurones.

Alternatively, the decreased parvocellular CRF mRNA levels may reflect the high mean daily circulating levels of glucocorticoids which occur in late pregnancy (Atkinson & Waddell, 1995). However, whether the free concentrations of corticosterone also increase is not known since the levels of corticosterone binding globulin has also been reported to increase with pregnancy (Seal & Doe, 1967). It is well documented that glucocorticoids act at extrahypothalamic sites to regulate the activity of the HPA axis. One of the best characterised is the hippocampus which sends projections to the CRF-synthesising neurones of the PVN via the BNST (Cullinan *et al.*, 1993) and expresses high levels of GR immunoreactivity (Fuxe *et al.*, 1985). Studies have shown that damage to the hippocampal formation or sectioning of the fimbria-fornix, another indirect pathway to the PVN, elicits hypersecretion of CRF, AVP, ACTH and corticosterone and increased expression of ACTH secretagogue mRNAs in the PVN, supporting the idea that the hippocampus plays an inhibitory role in the regulation of the HPA axis (Herman *et al.*, 1989, 1992; Sapolsky *et al.*, 1984,1989; Wilson *et al.*, 1980). Another limbic structure which sends projections to the parvocellular neurones via the BNST is the amygdaloid complex (de Olmos *et al.*, 1985; Sawchenko & Swanson, 1983) which also expresses high levels of GR immunoreactivity (Fuxe *et al.*, 1985). The amygdala has been shown to be involved in the regulation of the HPA axis (Beaulieu *et al.*, 1986;

Feldman & Conforti, 1981; Carrillo, 1977; Dunn & Whitener, 1986; Matheson *et al.*, 1971; Redgate & Fahringer, 1973). However, it seems to possess both stimulatory and facilitatory influences on ACTH and corticosterone secretion. Several lesion studies have demonstrated an excitatory effect on stress-induced ACTH secretion (Allan & Allan, 1974,1975; Beaulieu *et al.*, 1986) while other studies found that bilateral removal or electrolytic destruction of the amygdala results in enhanced adrenocortical activity (Martin *et al.*, 1958; Yamada & Greer, 1960) supporting an inhibitory role for this limbic structure. The amygdaloid complex actually consists of a number of subnuclei (for review see Price, 1981) and the stimulation of the separate subnuclei appears to have differing effects on plasma corticosterone concentrations (Dunn & Whitener, 1986). A recent report which examined the effect of lesions of different regions of the BNST on unstimulated CRF mRNA expression indirectly supports this finding. Lesions of the anterolateral BNST reduced basal CRF expression while damage to the posterior intermediate and/or posterior medial BNST increased CRF expression, thus reflecting the diverse limbic system inputs to the BNST (Herman *et al.*, 1994).

Numerous studies have demonstrated that corticosterone has direct effects on CRF expression in the PVN and GR are localised on parvocellular CRF neurones (Reul & de Kloet, 1985). Adx leads to an increase in CRF and AVP immunoreactivity in the PVN (Kiss *et al.*,1984; Sawchenko *et al.*, 1984) and this enhancement can be prevented by corticosterone implants around the PVN (Kovacs *et al.*, 1986). CRF gene expression can be inhibited when capsules containing the GR agonist, dexamethasone are implanted into the region of the PVN (Kovacs & Mezey, 1987). Glucocorticoid suppression of the CRF gene might be mediated through the interaction of the activated GR with *c-fos* or *c-jun* and inhibition of the formation of the AP-1 complex (Lee *et al.*, 1991; Lucibello *et al.*, 1990). Interestingly the human CRF gene appears to possess a perfect half glucocorticoid-response element (GRE) in its promoter region (Vamvakopoulos & Chrousos, 1993) and half-GREs have been shown to confer delayed secondary glucocorticoid responses to another gene product (Chan *et al.*, 1991; Slater *et al.*, 1993); however, in the promoter region of the rat CRF gene no GREs have so far been identified (Seaholtz *et al.*, 1988). If

glucocorticoids were acting directly at the PVN to decrease CRF gene expression during pregnancy we might also have expected to see a decrease in AVP mRNA expression. Parvocellular AVP-containing neurones are sensitive to inhibition by glucocorticoids. Removal of endogenous glucocorticoids by adx greatly increases the expression and median eminence content of AVP whereas adx does not consistently increase CRF mRNA (Albeck *et al.*, 1994; Holmes *et al.*, 1986) indicating that glucocorticoid feedback is able to restrict parvocellular PVN AVP mRNA levels more effectively than CRF mRNA expression. A recent report using reporter gene constructs demonstrated that the AVP gene promoter region was negatively regulated by glucocorticoids (Iwasaki *et al.*, 1997). However, as mentioned previously our finding of no significant change in AVP mRNA expression in the parvocellular PVN during pregnancy indicates that the reduced CRF mRNA expression in the parvocellular PVN during pregnancy is unlikely to be determined by glucocorticoids.

It is tempting to postulate that this decreased CRF mRNA expression in the parvocellular cell bodies of the PVN would result in a decreased storage pool of CRF in the median eminence and hence reduced release into the HPB upon stimulation and contributing to the decreased secretion of ACTH, and subsequently of corticosterone in response to an acute stress in late pregnancy (Neumann *et al.*, 1998 see Chapter 3). Consistent with this hypothesis is the study of da Costa *et al* (1996) which examined c-*fos* mRNA expression in response to an acute stress and found that lower levels of c-*fos* expression occurred in the PVN of the late pregnant rat. However, in order to determine conclusively that this decreased gene expression is related to the releasable store of CRF further studies would have to be carried out. One possibility is to use hypophysial blood sampling. Unfortunately, this procedure involves major surgery and therefore has to be done under anaesthesia thereby preventing the use of psychological stressors, such as restraint stress (Plotsky et al., 1987). In addition, certain anaesthetics appear to affect the release pattern of ACTH secretagogues into the HPB (Sherwood & Fink, 1991). Although mRNA analysis studies provide a good indication of the influence of chronic physiological challenge on gene transcription (Sherman *et al.*, 1986; Znigg *et al.*, 1986), the steady state level of mRNA in a neurone does not necessarily reflect rapid changes in gene

transcription and may not be the most sensitive way of detecting changes in stress responsiveness in pregnancy. An alternative hybridisation method would be to use probes directed towards intronic sequences of the gene thus allowing the detection of primary transcripts or heteronuclear RNA (hnRNA), and several studies have demonstrated that it provides an accurate temporal relationship between the acute stimulus and gene transcription (Fremeau *et al.*, 1986, 1989).

4.4.2 [^{125}I] oCRF autoradiography and *in vitro* cAMP accumulation

The decreased ACTH and corticosterone response *in vivo* to exogenous CRF (Neumann et el., 1998) may, in part, be explained by the significant decrease in [^{125}I]oCRF binding on day 10 and 16 and a further decrease on day 21 of pregnancy. However, in order to assess whether this decreased binding is due to a reduced Kd or a decrease in the number of binding sites (Bmax), studies using Scatchard analysis would have to be performed.

Several *in vivo* and *in vitro* studies have demonstrated that CRF possesses the ability to downregulate its own receptors. It is a classical mechanism in several endocrine systems which is considered to protect the target organ from over-exposure to its trophic factor. It is not known whether CRF HPB levels are higher during pregnancy. However, our CRF mRNA expression measurments do not support this as expression is significantly reduced during pregnancy.

Circulating glucocorticoids may be responsible for the decrease in pituitary CRF binding as they have been shown experimentally to decrease both the binding of CRF to anterior pituitary membranes (Hauger *et al.*, 1987,1990) and the levels of $CRFR_1$ mRNA (Makino *et al.*, 1995).

The cAMP data from the studies on both the pitutary segments and isolated anterior pituitary cells indicate that the corticotrophs are less responsive to CRF during late pregnancy which complements both the *in vivo* and the CRF receptor autoradiography data. However, a discrepancy does exist between the cAMP accumulation and the CRF binding data prior to day 21 of pregnancy. In the binding data we found a 50% decrease in the CRF binding on day 10 and 16 of pregnancy compared to virgins, however, exogenous CRF did not affect cAMP accumulation in

pituitary segments until day 21 of pregnancy. This is likely to be a result of the large CRF receptor capacity previously reported in corticotrophs (Antoni, 1986; King & Baertschi, 1990), however, additional changes occurring at the level of the adenylate cyclase complex cannot be ruled out. We demonstrated that this phenomenon of reduced corticotroph sensitivity in late pregnancy also occurs in acutely dispersed cells indicating that this reduced responsiveness was not a consequence of a cell to cell contact. The difference in the basal cAMP values between pregnant and virgin rats (significantly higher levels on day 21 of pregnancy) in the acutely dispersed cells was unexpected and not easily explained. It may instead, reflect the absence of IBMX in the cell preparation and indicate a difference in phosphodiesterase activity in pregnancy; alternatively dispersing the cells may lead to a removal of an autocrine/paracrine factor present in the anterior pituitary in pregnant animals which suppressed the basal cAMP accumulation. Studies on ACTH secretion from dispersed cells are needed.

In conclusion, parvocellular CRF mRNA expression, anterior pituitary CRF binding site density and *in vitro* CRF-stimulated cAMP accumulation all decreased in late pregnancy. These changes will affect the effectiveness of the forward drive, both centrally and at the anterior pituitary level, to the HPA axis and thus are likely to contribute to the hyporesponsiveness of the HPA axis to acute stress seen in pregnancy.

CHAPTER 5

The glucocorticoid negative feedback signal during pregnancy

5.1 Introduction

Glucocorticoids, corticosterone in rodents and cortisol in man, negatively regulate both the basal and stress-induced activity of the HPA axis in a closed loop manner. Under *in vivo* conditions at least three distinct time domains have been identified in which glucocorticoids exert a negative feedback signal on stimulated ACTH secretion: fast rate-sensitive; intermediate; and slow feedback (for review see Keller-Wood & Dallman, 1984).

The existence of fast, rate sensitive feedback was first postulated by Dallman & Yates (1969) and occurs within seconds to minutes of glucocorticoid exposure when the plasma levels of the hormone are rising. However the effects only last as long as the plasma levels are rising at a sufficient rate (Jones *et al.*, 1972). Although the mechanism of inhibition is not known the rapidity of the effects indicate that protein synthesis is not involved and in support of this, pretreatment of perifused pituitaries with cycloheximide, a protein synthesis inhibitor, does not prevent rapid inhibition (Widmaier & Dallman, 1983). Several *in vitro* studies have demonstrated that glucocorticoids have rapid effects on both stimulated CRF and ACTH secretion (Buckingham & Hodges, 1977; Vale & Rivier, 1977; Vermes *et al.*, 1977). While the early *in vivo* studies were based on administration of exogenous glucocorticoid and measurement of indirect indices of ACTH secretion (Dallman & Yates, 1969; Kaneko & Hiroshige, 1978; Yates *et al.*, 1972), a more recent study demonstrated physiological evidence for the rate-sensitive fast feedback inhibition of ACTH secretion (De Souza & Van Loon, 1989). They used a 2 min restraint stress paradigm to elevate corticosterone levels and then applied a second restraint during the period when the plasma corticosterone levels were significantly rising. They found that although the maximum plasma ACTH concentrations were not different the rate of decline was significantly greater following the second restraint. This indicates that

rate-sensitive feedback acts to terminate and limit the duration, but not the peak, of the ACTH response to subsequent stress.

Dallman & Yates (1969) also hypothesised the existence of a delayed feedback effect of corticosteroids on ACTH secretion which was dependent on the duration of exposure to elevated levels of corticosteroids. One report found that *in vitro* incubation of ATt-20 cells (a murine pituitary tumour cell line) with dexamethasone for 5-25 h caused a significant decrease in ACTH release, however, following 2-3 days of dexamethasone exposure, both the amount of ACTH released and the ACTH cell content were significantly reduced (Watanabe *et al.*, 1973). This led to the hypothesis that the glucocorticoid delayed feedback exerts different effects over time. Therefore, delayed feedback was divided into intermediate and slow feedback effects.

Intermediate feedback requires corticosterone exposure for 2-10 h previously and affects ACTH secretion but not synthesis. However, the mechanism of intermediate feedback on ACTH secretion does require DNA-dependent RNA and protein synthesis since the suppressive effects of the steroid are prevented in the presence of either actinomycin D, a RNA polymerase inhibitor, or cycloheximide (Arimura *et al.*, 1969; Philips & Tashjian, 1982). Intermediate feedback has also been shown to affect CRF synthesis and release (Buckingham, 1979; Sato., 1975).

Slow feedback by corticosteroids results from constant corticosterone exposure for 12 h or longer and inhibits both ACTH release and synthesis. *In vivo*, glucocorticoid injections reduce the pituitary POMC mRNA levels in adx rats (Nakanishi *et al.*,1977) and *in vitro*, dexamethasone, cortisol and corticosterone specifically inhibit mRNA coding for POMC without altering the synthesis of other proteins (Roberts *et al.*, 1979).

Several studies have used *in vitro* pituitary model systems; primarily rat corticotroph cells and AtT-20 cells, a murine pituitary tumour cell line which has been demonstrated to be a good model for studying the influence of various factors on the CRF second messenger pathway (Clark & Kemppainen, 1994). In contrast to the *in vivo* situation, the characteristics of feedback in these cell systems are similar regardless of whether glucocorticoids are applied for minutes to hours and therefore is termed 'early' feedback. It has been proposed that early feedback may be mediated

by inhibition of agonist-induced intracellular and/or extracellular Ca^{2+} transients (Antoni *et al.*, 1992; Link *et al.*, 1993). In corticotroph cells this early inhibition of ACTH release involves the induction of new mRNA and protein (Dayanithi & Antoni, 1989) similar to *in vivo* intermediate feedback. Three mechanisms have been proposed to account for the mechanism of action of proteins mediating early glucocorticoid feedback. Firstly, lipocortin I, the inhibitor of phospholipase A2, may mediate early glucocorticoid feedback (Flowers, 1988) since it is induced following glucocorticoid treatment in the anterior pituitary. Secondly, it has been hypothesised that glucocorticoids stabilise the actin cytoskeleton in the corticotrophs preventing the fusion of the ACTH secretory granules with the cell membrane and thereby exocytosis (Castillino *et al.*, 1992). Thirdly, calmodulin, the Ca^{2+} receptor protein may play a role in mediating the early feedback by buffering the internal Ca^{2+} concentrations (Epstein *et al.*, 1992). Consistent with this is the observation that dexamethasone treatment increases calmodulin mRNA levels in AtT-20 cells (Shipston & Antoni, 1992).

The influence of glucocorticoids on the activity of the HPA axis is predominantly mediated via intracellular corticosteroid receptors which act as ligand-bound transcription factors, binding to specific DNA sequences and acting to either induce or repress the rate of transcription. The exception is the fast rate-sensitive feedback which occurs too quickly to involve these intracellular receptors. However, it has been shown that these rate-sensitive steroid feedback sites are saturable (Jones *et al.*, 1972) so it is hypothesised that glucocorticoids exert this effect via membrane-bound receptors, although evidence for the existence of these steroid receptors is limited (Chen *et al.*, 1993; Towle & Sze, 1983).

Two intracellular receptor types have been identified, mineralocorticoid receptors (MR) or type I and glucocorticoid receptors (GR) or type II (for review see De Kloet, 1991) and these belong to the steroid/thyroid hormone receptor gene superfamily (Evans, 1988). GR and MR differ in both their central distribution and pharmacological profiles (Reul & de Kloet, 1985). GR is widely expressed throughout the central nervous system (CNS) in both neurones and glia (Ahima & Harlan, 1990; De Vellis *et al.*, 1974). The highest densities of binding sites are found in the hippocampus, cerebellum, amygdala, the hypothalamic paraventricular

nucleus, the ascending monoaminergic neurones of the brainstem, including the locus coeruleus and nucleus of the solitary tract (NTS), and in the anterior pituitary (Bertini *et al.*, 1989; Fuxe *et al.*, 1985). Therefore, GR is highly expressed in several of the postulated negative feedback sites for glucocortiocoids on the HPA axis. MR has a more discrete central distribution with the highest density of binding sites confined to several limbic system structures, including the hippocampus and septum and in some brain stem nuclei (Reul & de Kloet, 1985). However, *in situ* hybridisation studies have revealed a more uwidespread distribution, in particular detectable but low levels of MR mRNA expression in the PVN (Seckl *et al.*, 1993).

Studies using purified and recombinant receptors have demonstrated that GR and MR differ in affinity for ligands (Arriza *et al.*, 1987; Reul *et al.*, 1987). In the rodent, GR exhibits the highest affinity for synthetic glucocorticoids, such as dexamethasone, and a lower affinity for physiological glucocorticoids, such as corticosterone (Kd = 2.5-5.0 nM) and cortisol. MR, on the other hand, shows the highest affinity for corticosterone (Kd = 0.5 nM) and aldosterone, followed by cortisol. It also binds dexamethasone but with a much lower affinity (Luttge *et al.*, 1989).

Recent studies into the regulation of the HPA axis by physiological levels of corticosteroids have demonstrated that replacing adx rats with a low constant signal of corticosterone via s.c. implants prevented the elevation of morning plasma ACTH levels but is insufficient to normalise stress-induced ACTH secretion (Akana *et al.*, 1988). Measurement of the free corticosterone found the levels were close to the Kd of corticosterone for MR. In a subsequent study Dallman *et al* (1989a) showed that low levels of corticosterone provided tonically to adx rats prevented basal morning but not evening ACTH concentrations being higher than sham-adx rats. In addition, similar levels of corticosterone have been found to be sufficient to prevent the adx-induced increase in parvocellular CRF mRNA when examined in the nadir but not the peak of the circadian rhythm (Kwak *et al.*, 1993). The use of centrally administered MR and GR antagonists initially suggested that MRs controlled basal HPA activity, while GRs mediate the termination of the stress response (Ratka *et al.*, 1989). However, the requirement for more corticosterone to reduce HPA activity at the circadian evening peak suggested that a shift in the control of basal HPA axis

activity from MRs to GRs in the evening occurred (Dallman *et al.*, 1989a). A recent study by Bradbury *et al* (1994) further clarified the situation demonstrating that dexamethasone is only capable of reducing evening plasma ACTH levels in adx rats in the presence of low concentrations of corticosterone indicating that MR occupancy is necessary for GR-mediated effects on diurnal peak ACTH levels. In addition MRs appear to play a role in the termination of the stress response (Ratka *et al.*, 1989) since administration of a specific MR antagonist causes an elevated and prolonged corticosterone response to stress in a similar manner to that observed following administration of a GR antagonist. Therefore GR and MR appear to work cooperatively in the control of the basal and stress-induced activation of the HPA axis.

There is plenty of evidence to indicate that corticosteroids act at the anterior pituitary, hypothalamus and extrahypothalamic sites to regulate the activity of the HPA axis.The anterior pituitary as a locus for corticosteroid inhibition of the pituitary-adrenal responses to stress is supported by both *in vivo* and *in vitro* studies and by the fact that GR immunoreactivity occurs in POMC cells of the anterior pituitary (Bertini *et al.*, 1989). Systemic administration of dexamethasone has been shown by some investigators to decrease pituitary sensitivity to CRF preparations in animals with median eminence or hypothalamic lesions (Jones *et al.*, 1977; De Weid, 1964). Dexamethasone has also been shown to suppress the plasma corticosterone concentration in rats with forebrains, including the hypothalamus, median eminence and pituitary stalk removed (Dunn & Critchlow, 1969). *In vitro* corticosteroids inhibit stimulated ACTH release from incubated pituitaries (Buckingham, 1977), monolayer cultures of pituitary cells (Fleischer & Rawls, 1970), isolated pituitary cells (Portanova & Sayers, 1974), perfused pituitaries (Widmaier & Dallman, 1983) and AtT-20 cells (Herbert *et al.*, 1978). However, the relative importance of the pituitary as a feedback site under physiological conditions is difficult to assess since the majority of studies used the synthetic glucocorticoid, dexamethasone, which does not bind to the intracellular corticosterone binding globulin, transcortin, which is highly expressed in the anterior pituitary (De Kloet & McEwen. 1976; Koch *et al.*, 1976) where it is proposed to sequester corticosterone in the cell and prevent its actions. Therefore, these studies may not necessarily reflect the relative importance

of the anterior pituitary *in vivo*. Several studies have implicated the parvocellular CRF neurones of the PVN as a site for glucocorticoid feedback. An immunohistochemical study demonstrated that GR immunoreactivity was localised in the PVN CRF neurones (Agnati *et al.*, 1985). Local injections or implants of corticosterone or dexamethasone effectively suppress pituitary-adrenal activity when placed in the median eminence or anteromedial hypothalamus but not the pituitary (Bohus & Strashmirov, 1970; Stark *et al.*, 1968), indicating that the hypothalamus is more sensitive to corticosterone. Interestingly, CBG protein is almost absent from the adult rat brain (McEwen *et al.*, 1986). Glucocorticoids have also been shown to affect neural activity in the hypothalamus. Microionotophoresis of corticosterone altered the firing rate of some hypothalamic neurones (Mandelbrod *et al.*, 1974). However, a recent *in vitro* experiment using explanted hypothalamic fragments from monkeys showed that 6 h perfusion with 10^{-6} M dexamethasone did not alter either the amplitude or the frequency of the pulsatile CRF secretion (Mershon *et al.*, 1992). This indicates that glucocorticoids may act at extrahypothalamic sites proximal to the CRF neurones of the PVN. One of the best documented extrahypothalamic sites for glucocorticoid feedback is the hippocampus, which has created much interest mainly due to its high density of both GR and MR. A number of studies have indicated that the hippocampus can influence both the circadian rhythm as well as the stress-induced activation of the HPA axis. Lesions of the hippocampus or fornix, the major efferent pathway of the hippocampal formation, reduces the diurnal variation in plasma corticosterone by primarily raising the nadir level while the peak remains unchanged (Fischette *et al.*, 1980; Moberg *et al.*, 1971). However, in these studies the possibility of the lesion-induced rise in the morning corticosteroid levels inhibiting the increase in the circadian peak could not be ruled out. Subsequently, a study by Bradbury & Dallman (1989) demonstrated that hippocampal implants of the GR antagonist RU 38486 in adx rats replaced with constant corticosterone levels augment afternoon plasma ACTH levels. This indicates that the hippocampus influences both circadian morning and evening ACTH secretion. Several lesion studies corroborate an inhibitory influence of hippocampal inputs on the stress-induced responses of the HPA axis. Dorsal or complete hippocampectomy increases plasma corticosteroid responses to jugular vein cannulation under anaesthesia

(Feldman & Conforti, 1980) while destruction of more than 50% of hippocampal neurones with stereotaxic kainate infusion enhances the restraint stress-induced increases in plasma corticosterone and prolongs the response (Sapolsky *et al.*, 1984). However, in a recent study it was found that lesions of the fimbria-fornix did not enhance either the circadian rhythm or the stress-induced ACTH plasma levels (Bradbury *et al.*, 1993). The discrepancy between this and other studies may reflect incomplete lesioning, but in the latter study the completeness of the lesion was verified immunohistochemically. However, more recently a group identified an indirect efferent inhibitory pathway from the hippocampus to the PVN via the BNST (Cullinan *et al.*, 1993), thus this additional pathway may maintain the influence of the hippocampus after fornix transection. Other limbic brain areas which possess high densities of MR and GR are the septum and the amygdaloid complex both of which have been implicated in the control of ACTH release (Seggie, 1987; Yamada & Greer, 1960). Both inhibitory and stimulatory effects on plasma corticosterone have been demonstrated for the amygdala (Carrillo & Dunn, 1977; Yamada & Greer, 1960). The amygdaloid complex is composed of several individual nuclei which project differentially to the hypothalamus (Krettek & Price, 1978) and in the anaesthetised rat, Dunn & Whitner (1986) demonstrated that stimulation of the different amygdaloid nuclei had different effects on plasma concentrations of corticosterone.

The presence of both GR and MR in the brain encouraged investigators to examine whether the enzyme, 11β-hydroxysteroid dehydrogenase (11β-HSD) was expressed in areas of the brain where MR selectively binds aldosterone, such as in the circumventricular areas (McEwen *et al.*, 1986) and acts as a dehydrogenase as it does in the kidney to prevent occupation of aldosterone-selective MR by corticosterone (Edwards *et al.*, 1988; Funder *et al.*, 1988). However, it was found that the majority of hippocampal MR bound corticosterone and aldosterone with equal affinity *in vivo* and 11β-dehydrogenase bioactivity was not detected in whole hippocampal extracts *in vitro* (Edwards *et al.*, 1988; Funder *et al.*, 1988). This led to the suggestion that this specificity-conferring mechanism was absent in the brain. However, earlier studies had demonstrated that 11β-HSD bioactivity was present in whole brain extract in the presence of NADP (Peterson *et al.*, 1965). Subsequently,

the presence of 11β-HSD in the brain was determined immunohistochemically (Sakai et al., 1992) and *in situ* hybridisation studies demonstrated that 11β-HSD1 possessed a widespread central distribution (Moisan *et al.*, 1990b). Interestingly, high enzyme activity and mRNA expression were detected in the hippocampus, anterior pituitary and in a separate study using a non-radioactive probe in both magnocellular and parvocellular neurones of the PVN (Seckl *et al.*, 1993). This suggested a role in the regulation of the glucocorticoid feedback signal. *In vitro* this enzyme interconverts corticosterone to 11-dehydrocorticosterone in rodents and cortisol to cortisone in man, however, there is increasing evidence from using cell lines (Agarwal *et al.*, 1990; Duperrex *et al.*, 1993; Low *et al.*, 1994a) and primary cultures of hepatocytes (Jamieson *et al.*, 1995) and fetal hippocampal neurones (Rajan *et al.*, 1996) that suggests *in vivo* 11β-HSD1 acts almost exclusively as a reductase, to reactivate, rather than to inactivate, glucocorticoids. The highest levels of 11β-HSD1 mRNA expression and bioactivity occur in brain areas which express high levels of GR, such as the cerebellum and hippocampus (Moisan *et al.*, 1990a, 1990b) and it has been proposed that a major role of 11β-HSD1 is to modulate corticosteroid access to GR rather than to MR (Moisan *et al.*, 1990b; Monder & Lakshmi., 1990; Teelucksingh *et al.*, 1990; Whorwood *et al.*, 1992).

11β-HSD1 is an inducible enzyme and several *in vivo* and *in vitro* studies have demonstrated that it is regulated by a number of different hormones, including glucocorticoids (Low *et al.*, 1994b) , thyroid hormone, insulin (Whorwood *et al.*, 1993), estradiol and growth hormone (Low *et al*, 1993, 1994c).

In this study we investigated whether the attenuated neuroendocrine responses of the HPA axis to acute stress were due to enhanced sensitivity of the HPA axis to the glucocorticoid negative feedback signal. Firstly, we used *in situ* hybridisation techniques to determine whether an upregulation in the expression of GR and MR mRNA in the subfields of the hippocampus or in the parvocellular neurones of the PVN occurred during pregnancy. Secondly, we measured *in vitro* 11β-HSD1 bioactivity in the main glucocorticoid feedback sites; thirdly we examined the effect of inhibiting central 11β-HSD *in vivo* with the potent inhibitor, glycyrrhetinic acid (GA) on the HPA axis responses to forced swim stress. GA is the active component of liquorice and *in vivo* is a potent inhibitor of 11β-HSD. Following administration it

reveals both renal and colonic actions of cortisol (Stewart *et al.*, 1987) and produces a binding pattern of [^{3}H]corticosterone in the rat kidney which is indistinguishable from [^{3}H]aldosterone (Edwards *et al.*, 1988). *In vitro* it has been shown to be a potent inhibitor of central 11β-HSD activity (Moisan *et al.*, 1990a) and 11β-HSD1 mRNA levels in rat pituitary cells (Whorwood *et al.*, 1993).

Finally, we tested the sensitivity of the whole axis *in vivo* by chemically adrenalectomising pregnant and virgin rats with metyrapone, a 11β-hydroxylase inhibitor which prevents the conversion of 11-deoxycorticosterone to corticosterone in the rat, and aminoglutethimide, the 20α-hydroxylase inhibitor which interrupts the synthesis of all steroids as it prevents the conversion of cholesterol to pregnenolone (Plotsky & Sawchenko, 1987).

5.2 Materials and Methods

5.2.1 GR and MR *in situ* hybridisation

For details see Chapter 2. Briefly, individually housed day 10, 16 & 21 pregnant and virgin rats were transferred separately to the experimental room where they were decapitated between 09.30 h and 10.30 h. The brains were rapidly removed and immediately frozen on dry ice. 20 μm cryostat-cut coronal brain sections mounted onto gelatin and poly-L-lysine coated slides were fixed in 4% paraformaldehyde, incubated with prehybridisation buffer for 2 h at 50 °C and then hybridised overnight at 50 °C with ^{35}S-labeled antisense cRNA probes.

T7 polymerase was used to transcribe the GR riboprobe from an Ava I-linearised pGEM3 plasmid containing the rat GR cDNA insert, and SP6 polymerase was used to transcribe the MR riboprobe from a Hind III-linearised pGEM4 plasmid containing the rat MR cDNA insert.

The following morning the slides were washed in reducing salt concentration to a maximum of 0.1 x SSC at 60 °C for 60 min. Then the sections were dehydrated with increasing concentrations of ethanol in 0.3 M ammonium acetate, air-dried and placed against film for 7 days. Afterwards they were dipped in photographic emulsion and stored at 4 °C for 21 days before being developed and counterstained with pyronin Y.

To determine the specificity of the hybridisation signal adjacent slides were hybridised with ^{35}S-labelled sense cRNA probes which were not complementary to the GR or MR mRNAs. The GR and MR sense probes were transcribed using SP6 and T7 polymerase, respectively.

Analysis was carried out using a high power microscope (objective x40) linked to a computer-based image analysis system. Silver grains overlying cells indicated the presence of mRNA. For the GR *in situ* hybridisation study the number of silver grains per neurone was determined in the parvocellular PVN and the subfields of the hippocampus (CA1, CA3, CA4 & dentate gyrus, DG) and for MR mRNA expression the CA1, CA2, CA3, CA4 and DG hippocampal subfields were analysed; for each animal measurements were made on three sections. Background measurements were made on adjacent areas of tissue and automatically subtracted from the PVN or hippocampal measurements. The group means were then calculated and the data expressed as percentages of the virgin control.

5.2.2 11β-HSD1 activity *in vitro* and *in vivo*

5.2.2.a *In vitro* 11β-HSD1 bioactivity in the anterior pituitary, PVN and hippocampus.

See Chapter 2 for details. Briefly, on the day of the experiment separately housed day 16 & 21 pregnant and virgin rats were decapitated between 9.00 h and 10.00 h for tissue harvest.

For the assay of 11β-dehydrogenase activity (conversion of corticosterone to 11-dehydrocorticosterone) anterior pituitary, micropunched PVN and hippocampus from vibroslice brain sections, and part of the outer cortex of the cerebellum were collected into tubes on ice and homogenised in ice-cold buffer C (pH 7.7). Following colorimetric protein determination, PVN, anterior pituitary, hippocampus and cerebellum homogenates were incubated at a protein concentration of 1000 μg/ml, 500 μg/ml, 200 μg/ml and 100 μg/ml, respectively with 12 nM [^{3}H]corticosterone and 200 mM NADP for 60 min at 37 °C. For each PVN measurement tissue was pooled from two animals. For the assay of 11β-reductase activity (conversion of 11-dehydrocorticosterone to corticosterone) anterior pituitary and hippocampal

homogenates at the same protein concentrations as above were incubated with [^{3}H]11-dehydrocorticosterone and 200 mM NADPH for 10 min at 37 °C.

The resulting steroids were then extracted into ethyl acetate, dried down and reconstituted with ethanol containing cold corticosterone and 11-dehydrocorticosterone, to allow the visualisation of the separate steroid bands, and run on TLC plates. The bands were scraped into scintillant-containing vials and the radioactivity measured in a β-counter. 11β-dehydrogenase activity was expressed as % conversion of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone and 11β-reductase activity was expressed vice versa.

5.2.2.b *In vivo* inhibition of 11β-HSD1

This study was a collaboration with Prof. R Landgraf's group at the Neuroendocrinology laboratories at the Max Planck Institute of Psychiatry, Munich, Germany.

On the same day virgin day and pregnant Sprague Dawley rats underwent surgery under halothane anaesthesia to implant chronic intracerebroventricular (i.c.v) and jugular cannulae.

5.2.2.b.1 Intracerebroventricular (ICV) cannulation surgery

Under halothane anaesthesia and using sterile procedures day 15 pregnant and virgin rats were implanted with an ICV cannula for acute injections into the right lateral cerebral ventricle.

The scalp was shaved and the animal placed into a stereotaxic frame. A midline incision was made along the scalp and subcutaneous tissue gently scraped away from the skull surface. The needle pointer in the holder of the stereotaxic frame was then placed on bregma, the cruciform junction of bone plates in the the middle of the top of the skull, and using the Vernier scale on the frame the needle point was moved 0.6 mm posterior and 1.6 mm lateral to bregma. A 2 burr (1 mm diameter) was used to drill a hole in the skull at this site and the cannula fitted onto the end of the stereotaxic needle and lowered into place. In addition two holes close to the cannula hole were drilled using a 3 burr (1.5 mm diameter) and support screws inserted. The ICV cannula was then fixed in place with dental acrylic and once this had hardened

the scalp was sutured and a dummy cap placed on the cannula to seal it. The animals were allowed three days to recover from the surgery before the first i.c.v injection.

5.2.2.b.2 Chronic jugular vein cannulation

See Chapter 2 for details. Briefly, under halothane anaesthesia and using sterile procedures, day 15 pregnant and virgin rats were implanted with a chronic jugular vein catheter six days prior to the stress experiment.

The right jugular vein was exposed and a 4 cm silicone tubing cannula (Dow Corning, USA) connected to PE-50 polyethylene tubing containing sterile heparinised saline (heparin 20 IU/ml 0.9% saline) was inserted approximately 3 cm into the vessel until the tip reached the right atrium of the heart and exteriorised dorsally in the cervical region. The cannula was then filled with 0.9% sterile saline containing gentamycin (30000 IU/ml, Centravet, Germany) and flushed with the same solution three days after surgery. The rats were then housed singly and handled each day to familiarise them with the blood sampling procedure and to reduce non-specific stress responses during the experiments.

5.2.2.b.3 i.c.v. injection of 18β-glycyrrhetinic acid (GA), inhibitor of 11β-HSD

Day 19 pregnant and virgin rats were infused i.c.v. over 2 min with either GA (5 μg/5 μl) or vehicle (10% ethanol in sterile saline) at 07.00h and 18.00 h. The following day the infusions were repeated. The dose of GA to give i.c.v. was derived from a previous study where the drug had been administered s.c. (Seckl et al., 1993).

On the morning of day 21 of pregnancy the rats received their final infusion of GA or vehicle at 07.00 h. At 08.30 h sampling cannulae were connected and the animals left undisturbed for 90 min. At 10.00 h basal blood samples were collected and then each animal was exposed to 90 s of forced swim (FS) stress (clear plastic bucket, filled with tap water (19 °C) to a depth of approximately 40 cm). After the swim the rats were gently dried with towels for 10 s and returned to their home cages. Then 5, 15 and 50 min after the end of the stress blood samples were collected. The volume of each sample was 0.3 ml and each was immediately replaced by an equal volume of sterile 0.9% saline.

At the end of the experiment to check that the tip of the i.c.v. cannula lay in the cerebroventricular system 5 μl of coomassie blue dye was infused into the conscious animal. It was then killed by halothane overdose and the brain removed and sectioned by hand at the level of the implantation and the distribution of the dye assessed. In addition the pregnancy status of each animal was determined.

All blood samples were collected on ice in EDTA-coated tubes containing 10 μl aprotinin (Dayer AG, Leverksem, Germany) and centrifuged at 4 °C at 5 000 rpm for 5 min. Then plasma was divided into 80 μl aliquots for ACTH and 30 μl for corticosterone measurements.

Plasma ACTH and corticosterone concentrations were determined by specific and sensitive commercially available kits (ICN, USA) according to the respective protocols (Neumann *et al*, 1998).

5.2.3 Pharmacological adrenalectomy (adx) with metyrapone

Individually caged day 16 pregnant and virgin control rats each had a silastic cannula inserted into the right jugular vein by the method described in detail in Chapter 2 and were allowed to recover for four days prior to experimentation.

At 07.00 h, day 20 pregnant and virgin rats had sampling cannulae connected and were left undisturbed for 90 min then a basal blood sample (0.3 ml) was collected to assess pre-drug ACTH and corticosterone levels. Pharmacological adx was achieved by a modification of the method of Plotsky & Sawchenko (1987). Metyrapone, an inhibitor of 11β-hydroxylase, which prevents the conversion of 11-deoxycorticosterone to corticosterone, (100 mg/kg) or saline (vehicle) was administered s.c. three times over a 24 h period.

On the morning of the experiment sampling cannulae were connected at 07.00 h and a blood sample collected 60 min later. Immediately following the blood sample the last metyrapone injection was given and 75 min later aminoglutethimide, an inhibitor of 20α-hydroxylase which thus prevents the conversion of cholesterol to pregnenolone, (200 mg/kg) was given s.c. and then at 10.00 h a third blood sample was collected. Corticosterone (2 mg/kg) was administered s.c. then at 15, 30 and 60 min post-injection blood samples were taken. A blood volume of 0.3 ml was collected at each time point and immediately substituted by an equal volume of

sterile 0.9% saline. At the end of the experiment the pregnancy status of each animal was checked.

All blood samples were collected into EDTA-containing tubes on ice and centrifuged at 3000 g for 5 min. The plasma was divided into aliquots of 100 µl for ACTH and 50 µl for corticosterone and stored at -80 °C and -20 °C, respectively.

Plasma concentration of ACTH was measured using a highly sensitive, specific commercially available two-site radioimmunometric assay kit (Euro-diagnostics, Netherlands) and corticosterone was measured by a specific scintillation proximity assay. For details see Chapter 2.

5.3 Results

5.3.1 GR and MR basal mRNA expression

In all the *in situ* studies the hybridisation signal was primarily located in the cytoplasm of the cells. We found the highest density of GR hybridisation occurred in CA1 subfield and the DG while the MR hybridisation was seen in the CA2 subfield. Hybridisation with the sense probes in adjacent sections did not produce a signal.

5.3.1.a GR mRNA expression in the parvocellular PVN

Silver grain density in the parvocellular PVN did not differ significantly between the virgin and pregnant rats or change across pregnancy (Fig 5.1a).

5.3.1.b GR mRNA expression in the hippocampal subfields

Silver grain density did not significantly differ in the CA1, CA3, CA4 or DG hippocampal subfields between virgin and pregnant rats (Fig 5.1b). However, there was a trend towards increased expression across pregnancy in the DG with a significant increase occurring on day 21 of pregnancy ($p < 0.05$ vs. day 10 of pregnancy).

5.3.1.c MR mRNA expression in the hippocampal subfields

Silver grain density did not significantly differ in the CA1, CA2, CA3, CA4 or DG hippocampal subfields between virgin and pregnant rats or across pregnancy (Fig 5.1c).

5.3.2 *In vitro* 11β-HSD1 activity in the anterior pituitary, PVN and hippocampus

5.3.2.a 11β-dehydrogenase activity

We found the highest enzyme activity in the cerebellum followed by the hippocampus and that this activity did not change during pregnancy (Fig 5.2a).

In contrast, activity in the homogenates from the anterior pituitary significantly increased between day 16 and day 21 of pregnancy ($p < 0.05$, n = 10-12 determinations per group).

However, the greatest change occurred in the homogenates from the PVN where activity approximately doubled between virgins and day 16 of pregnancy ($p < 0.05$, n = 4-5), with a further increase on day 21 of pregnancy ($p < 0.05$, n = 6).

5.3.2.b 11β-reductase activity

The % conversion of [^{3}H]11-dehydrocorticosterone to [^{3}H]corticosterone in hippocampal and anterior pituitary homogenates was low and did not significantly differ between pregnant and virgin rats (Fig 5.2b).

5.3.3 The effect of GA on the HPA axis secretory response to FS stress

Two way ANOVA for RM of the plasma ACTH and corticosterone levels in all groups demonstrated a significant interaction between time and group ($p < 0.0001$); all subsequent comparisons were derived from Newman-Keuls test ($p < 0.05$ indicates significance).

The basal plasma levels of ACTH fell below the detection limit of the assay (< 25 pg/ml) in all groups (Fig 5.3a). Five min after the end of the 90 s FS stress the

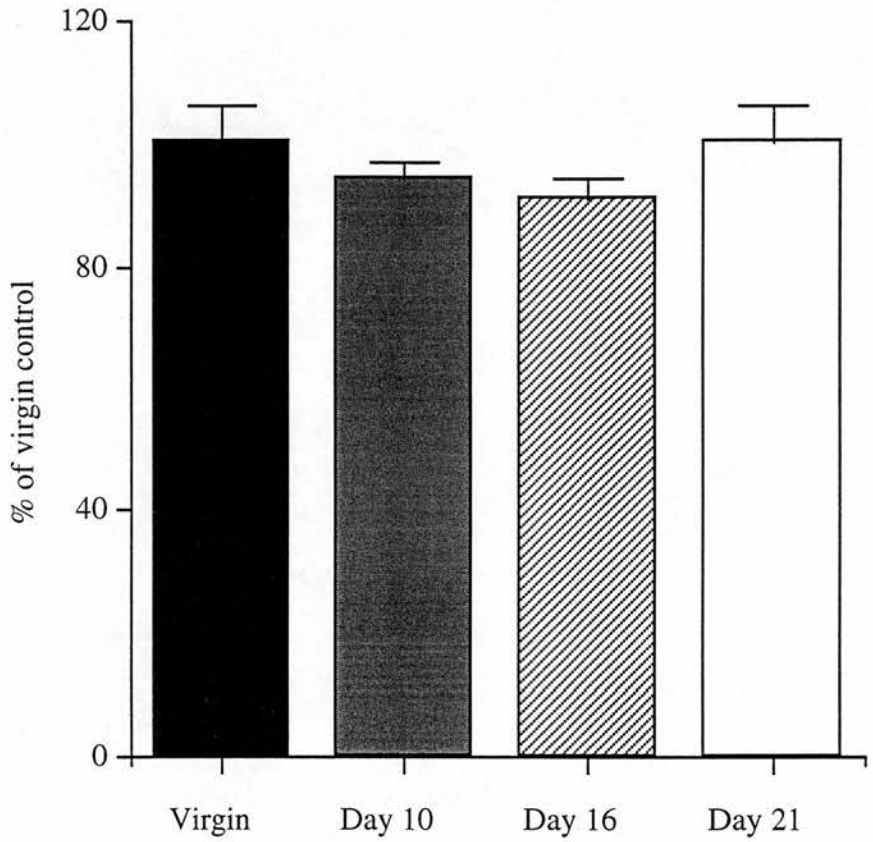


Fig 5.1a: GR mRNA expression in the parvocellular neurones of the PVN in virgin, day 10, day16 & day 21 of pregnancy. 20 μm coronal brain sections were hybridised with a ^{35}S-labeled GR cRNA probe over night. Sections were analysed by a computer-based image analysis system and the data expressed as % of virgin control (n = 6/group, mean ± S.E.M.)

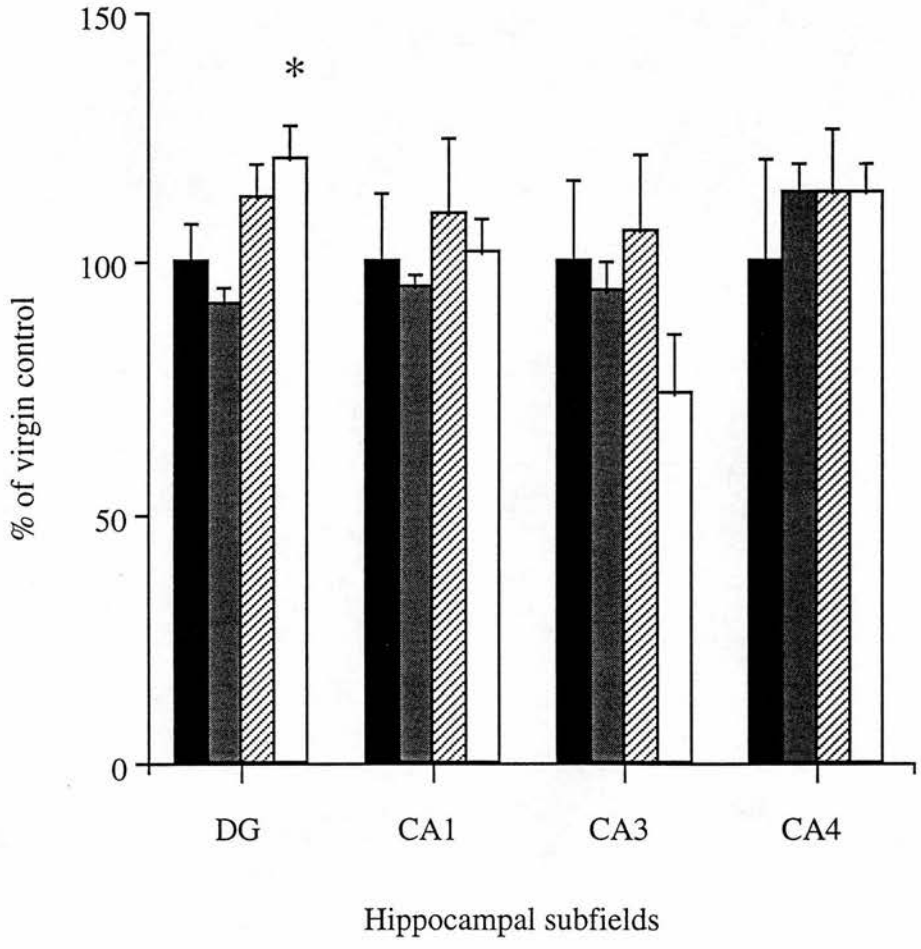


Fig 5.1b: GR mRNA expression in the hippocampus of virgin (■), day 10 (▩), day 16 (▨) & day 21 (□) of pregnancy. 20 µm coronal brain sections were hybridised with a ^{35}S-labeled GR cRNA probe overnight. Sections were analysed by a computer-based image analysis system and the data expressed as % of virgin control (n = 6/group, mean ± S.E.M.). * $p < 0.05$ vs. day 10 of pregnancy; ANOVA followed by Newman-Keuls student's test on raw data.

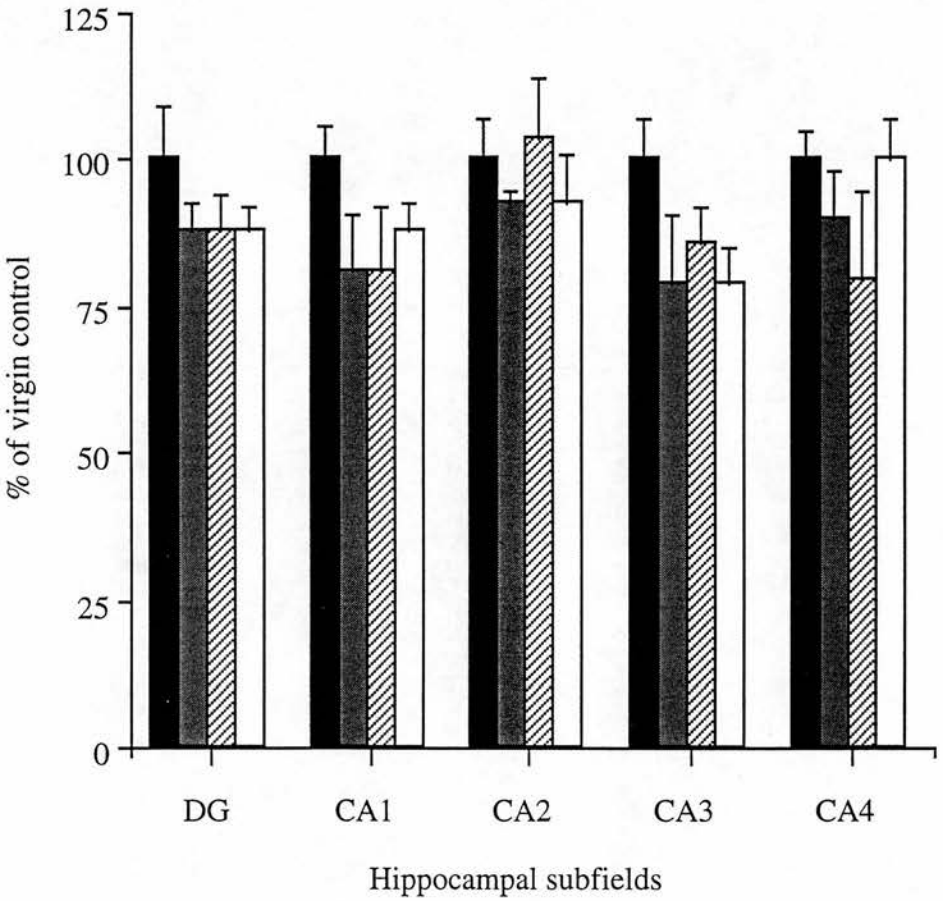


Fig 5.1c: MR mRNA expression in the hippocampus of virgin (■), day 10 (▩), day 16 (▨) & day 21 (□) of pregnancy. 20 µm coronal brain sections were hybridised with a ^{35}S-labeled MR cRNA probe overnight. Sections were analysed by a computer-based image analysis system and the data expressed as % of virgin control (n = 6/group, mean ± S.E.M.).

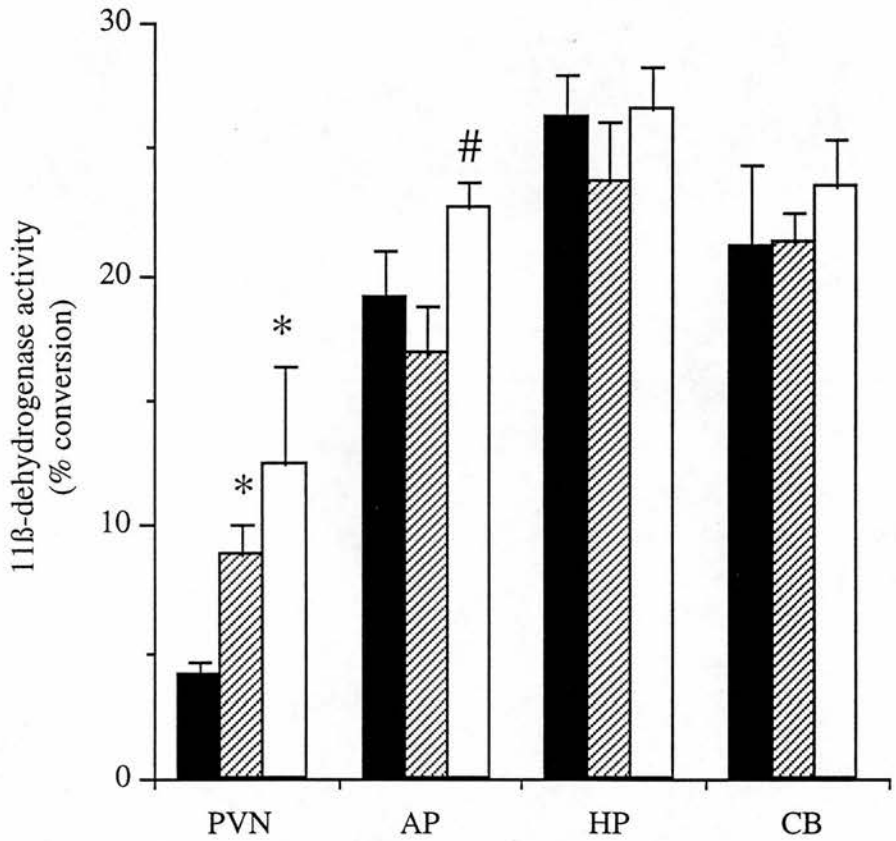


Fig 5.2a: 11β-dehydrogenase activity in homogenates of PVN micropunches (PVN), anterior pituitary (AP), hippocampus (HP) & cerebellum (CB) from virgin (■), day 16 (▨) & day 21 (□) pregnant rats. Brain tissue homogenates were incubated with [^{3}H]corticosterone and NADP at 37 °C for 60 min and steroids extracted into ethyl acetate and separated by TLC. Data are expressed as % conversion of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone. * $p < 0.05$ vs. virgin control, (n = 4-5 determinations for day 16 & n = 6 determinations for day 21 of pregnancy) & # $p < 0.05$ vs. day 16 of pregnancy, (n = 10-12 determinations). Kruskal-Wallis followed by a Mann-Whitney U test.

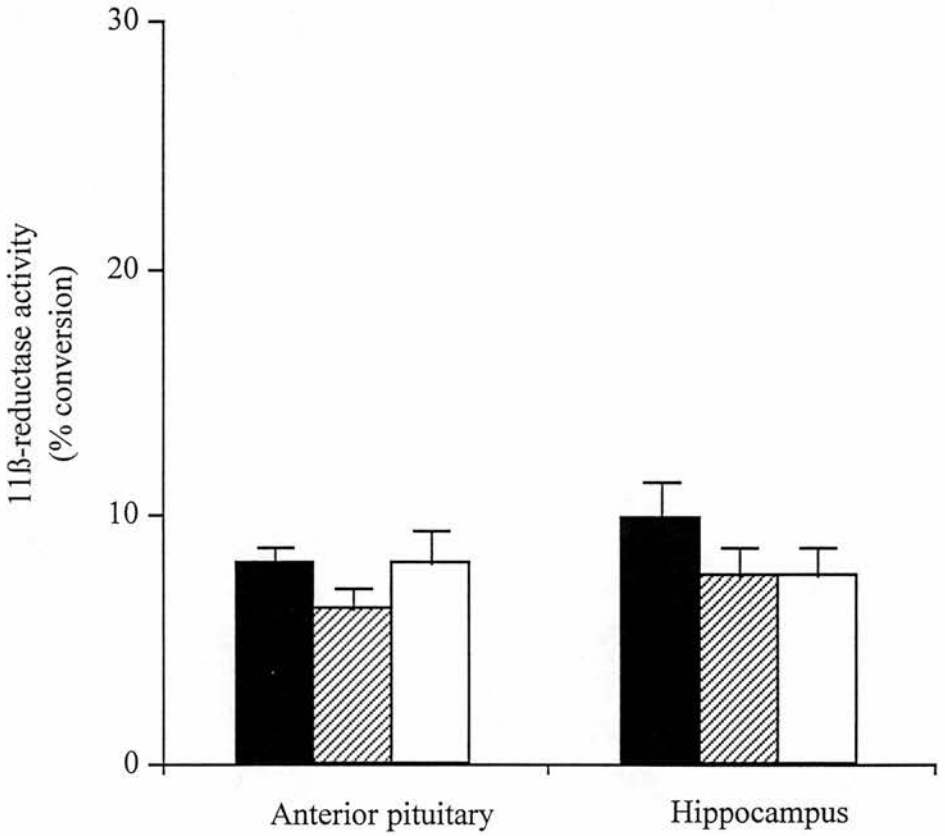


Fig 5.2b: 11β-reductase activity in homogenates of anterior pituitary (AP), hippocampus (HP) from virgin (■), day 16 (▨) & day 21 (□) pregnant rats. Brain tissue homogenates were incubated with [^{3}H]11dehydrocorticosterone and NADPH at 37 °C for 10 min and steroids extracted into ethyl acetate and separated by TLC. Data are expressed as % conversion of [^{3}H]11-dehydrocorticosterone to [^{3}H]corticosterone, n = 5-6 determinations .

ACTH levels were significantly lower in the GA-treated pregnant group compared to the GA-treated virgin group ($p < 0.05$) and in the vehicle-treated pregnant versus the vehicle-treated virgin group ($p < 0.05$). The peak ACTH response occurred at 5 min post-stress in all groups. At 15 min post stress the ACTH levels in both the GA-treated and vehicle-treated pregnant rats were still significantly lower then in the GA-treated and vehicle-treated virgin rats, respectively, ($p < 0.05$). Although the plasma ACTH levels in the GA-treated rats were consistently lower than the levels in the vehicle-treated pregnant rats at 5 & 15 min post-stress this was not statistically significant. By 50 min post-stress the plasma ACTH levels had returned to basal and were not significantly different between groups.

The basal corticosterone levels were not significantly different between groups (Fig 5.3b). Exposure to 90 s of FS stress significantly increased the plasma concentrations by 5 min in all groups ($p < 0.05$ vs. pre-stress level in respective groups) and by 15 min post-stress the maximum corticosterone concentrations were reached in all groups ($p < 0.05$). At 50 min post-stress the plasma corticosterone levels in both pregnant groups remained elevated compared to their basal values ($p < 0.05$) while the levels had returned to baseline in both virgin groups. The plasma corticosterone concentrations of the GA-treated and vehicle-treated pregnant groups were also significantly elevated compared to the vehicle-treated virgin group at 50 min post-stress ($p < 0.05$).

5.3.4 Feedback sensitivity of the HPA axis *in vivo*

Two way ANOVA for RM of the plasma ACTH and corticosterone levels in all groups demonstrated a significant interaction between group and time ($p < 0.0001$); all subsequent comparisons were derived from Newman-Keuls test ($p < 0.05$ indicates significance) unless otherwise stated.

The basal ACTH concentration prior to the metyrapone injections did not differ between groups (Fig 5.4a). Following the metyrapone/aminoglutethimide (met/amino) treatment the ACTH plasma levels were significantly increased. Twenty four hours after the first injection of metyrapone the plasma ACTH levels had risen

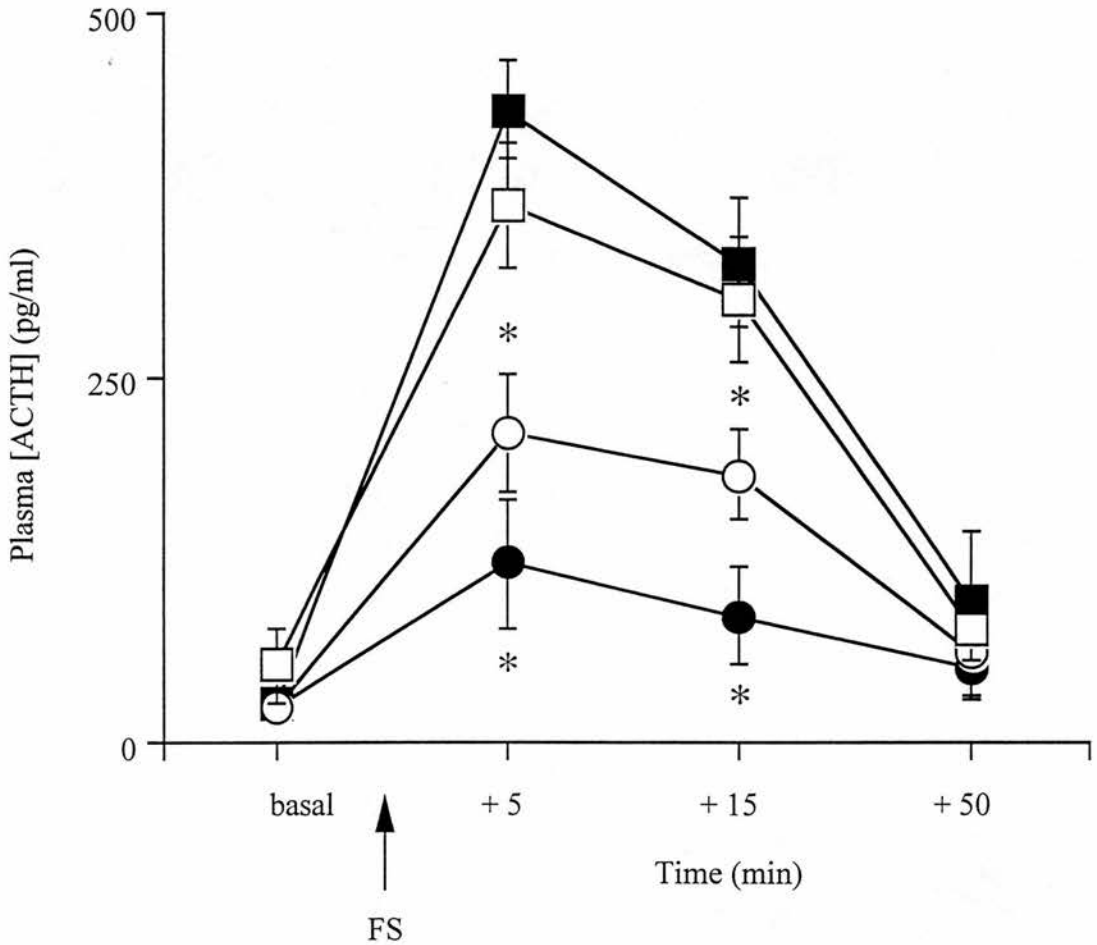


Fig 5.3a: The effect of glycyrrhetinic acid (GA) on the ACTH secretory response to forced swim (FS) stress. GA or vehicle (10% ethanol in saline) was administered by intracerebroventricular infusion every 12 h (07.00 & 19.00 h) for 48 h. Pregnant: GA (●, n = 7) & vehicle (○, n = 6) and virgin: GA (■, n = 8) & vehicle (□, n = 8).
On the morning of the experiment the last i.c.v. infusion was given at 07.00 h and 90 min later a basal blood sample was collected. Then the animals were exposed to 90 s of FS stress and blood samples collected 5, 15 & 50 min after the end of the stress.
GA-treated and vehicle-treated pregnant groups * $p < 0.05$ vs. GA-treated and vehicle-treated virgin groups, respectively. Two way ANOVA for RM followed by Newman-Keuls student's test.

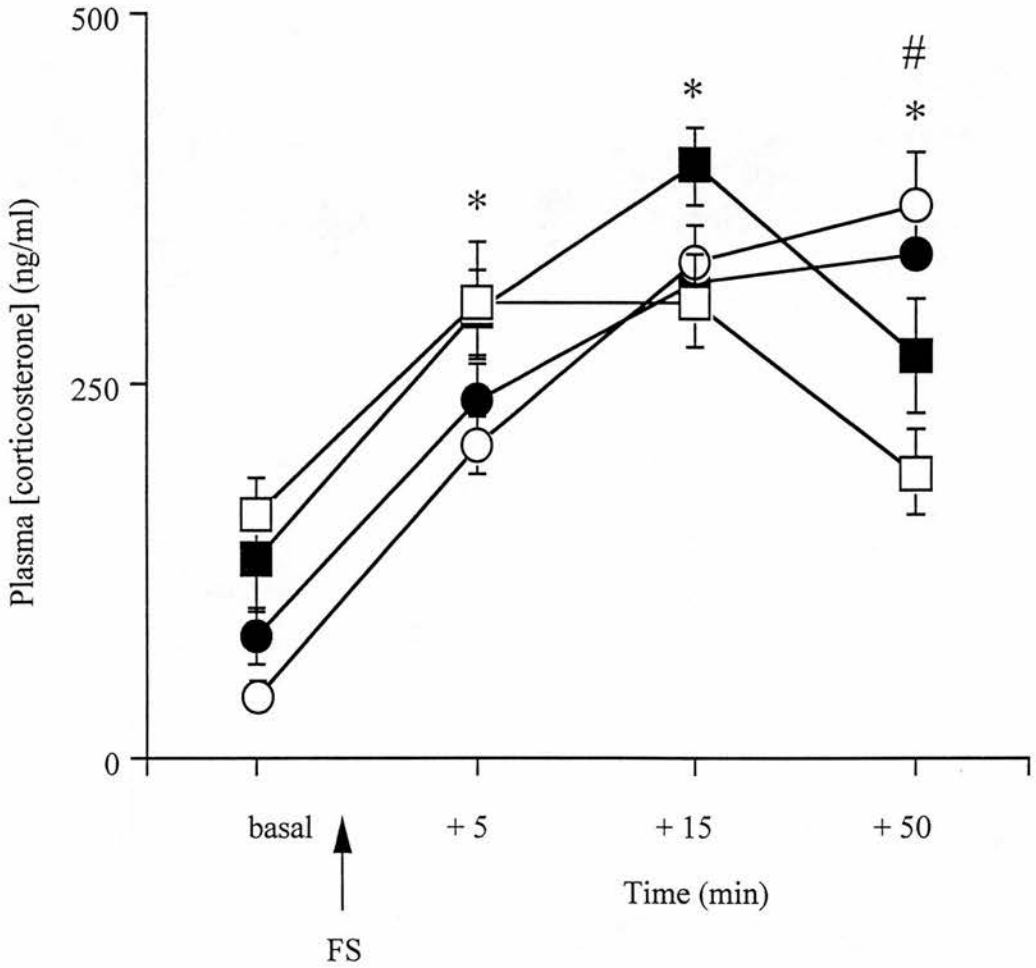


Fig 5.3b: The effect of glycyrrhetinic acid (GA) on the corticosterone secretory response to forced swim (FS) stress. GA or vehicle (10% ethanol in saline) was administered by intracerebroventricular infusion every 12 h (07.00 & 19.00 h) for 48 h. Pregnant: GA (●, n = 7) & vehicle (○, n = 6) and virgin: GA (■, n = 8) & vehicle (□, n = 8).
On the morning of the experiment the last i.c.v. infusion was given at 07.00 h and 90 min later a basal blood sample was collected. Then the animals were exposed to 90 s of FS stress and blood samples collected 5, 15 & 50 min after the end of the stress.
* $p < 0.05$ vs. all groups vs. their respective pre-stress levels & # $p < 0.05$ both pregnant groups vs. vehicle-treated virgin group. Two way ANOVA for RM followed by Newman-Keuls student's test.

10- and 12-fold in the pregnant and virgin groups, respectively ($p < 0.05$ vs. pre-met levels). There were no differences between the pregnant and virgin groups after met/amino. Exogenous corticosterone time-dependently decreased plasma ACTH levels. By 30 min post-injection the ACTH plasma concentration in the met-treated virgin group was significantly lower than the pre-corticosterone levels ($p < 0.05$) while in the met-treated pregnant group plasma ACTH was not significantly decreased. However, by 60 min post-injection the ACTH levels of the pregnant group was significantly lower than the pre-corticosterone level ($p < 0.05$). The plasma ACTH levels did not significantly change in the vehicle-treated pregnant and virgin groups throughout the experiment.

The pre-met corticosterone levels were not significantly different between groups (Fig 5.4b). Following met/amino treatment the plasma corticosterone levels decreased . Comparison of the change i.e decrement, between the pre-met and post-amino levels demonstrated a significant decrease in the drug-treated groups compared to the vehicle-treated groups ($p < 0.05$, Students t test). Following exogenous corticosterone the plasma levels significantly increased (20-fold and 4-fold in the drug-treated and vehicle-treated groups, respectively, $p < 0.05$). At 60 min post-injection plasma levels of corticosterone remained elevated in all groups compared to pre-drug levels ($p < 0.05$).

5.4 Discussion

5.4.1 GR and MR mRNA expression in the PVN and hippocampus

In some peripheral tissues the glucocorticoid/GR complex binds to the GR gene and potently downregulates its transcription and translation (Burnstein *et al.*, 1991), however, at other peripheral sites glucocorticoids upregulate GR, including in both T- and B-lymphocytes (Eisen *et al.*, 1988). Therefore, glucocorticoid effects on GR expression appear to be site-specific. Several studies have shown that central GRs are downregulated by exposure to high levels of glucocorticoids (Reul *et al.*, 1987) and upregulated 1-3 days after adx in the hippocampus (Reul *et al.*, 1989) while MRs in the hippocampus are upregulated by adx (Reul *et al.*, 1987). Interestingly, hippocampal MR mRNA expression is further upregulated by

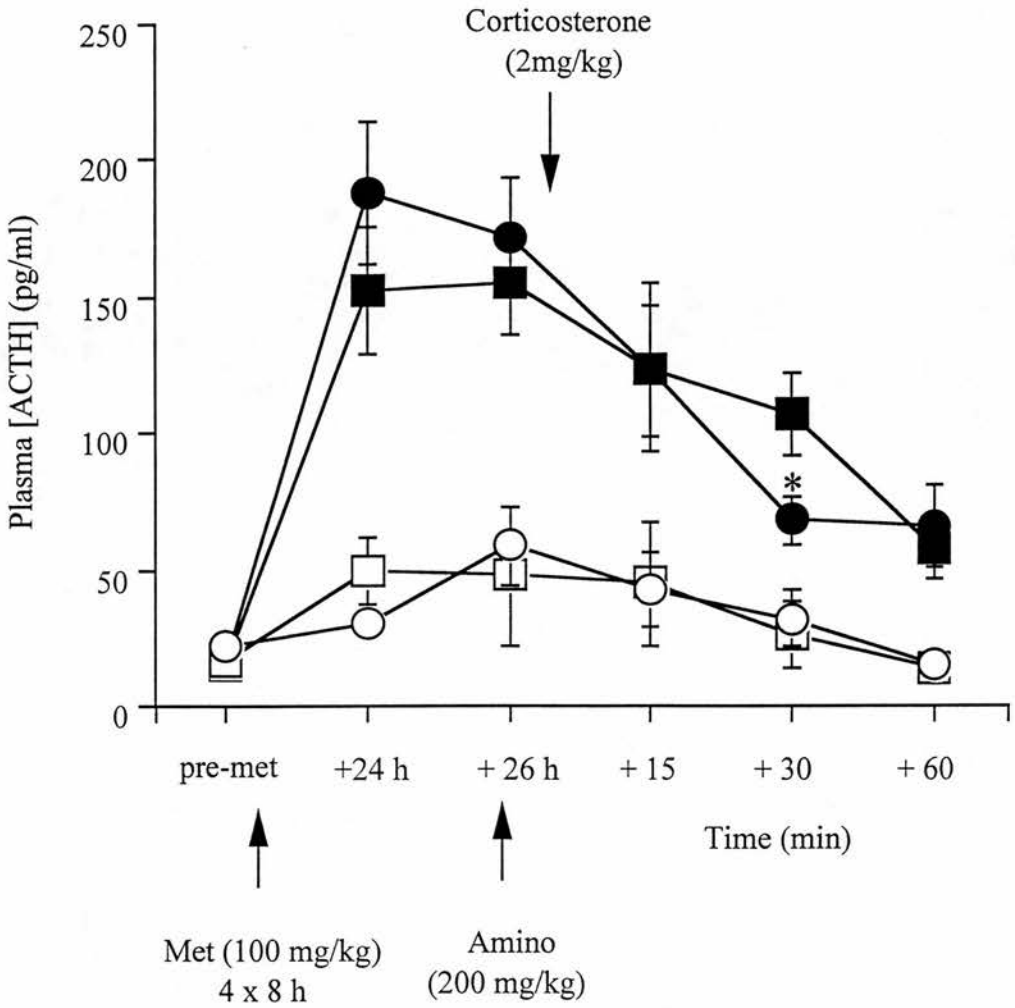


Fig 5.4a: The effect of exogenous corticosterone on the elevated plasma ACTH levels following pharmacological adrenalectomy. A basal blood sample was taken prior to the drug treatment, then the animals were given a subcutaneous (s.c.) injection of either metyrapone (met, 100 mg/kg), the 11β-hydroxylase inhibitor or vehicle (0.9% saline) every 8 h over 24 h. On the morning of the experiment another blood sample was collected followed by the final met injection at 08.00 h. 75 min later a s.c. injection of either aminoglutethimide (amino, 200 mg/kg) , the 20α-hydroxylase inhibitor or vehicle (DMSO) was given then after 45 min later a third basal sample was collected. Immediately afterwards a s.c. injection of corticosterone (2 mg/kg) was given and blood samples collected 15, 30 & 60 min post-injection. Day 21 pregnant: met/amino (■, n = 5) & vehicle (□, n = 3) and virgin: met/amino (●, n = 5) & vehicle (○, n = 5). Statistical analysis: two way ANOVA for RM followed by Newman-Keuls test, * $p < 0.05$ vs. + 26 h sample met/amino virgin.

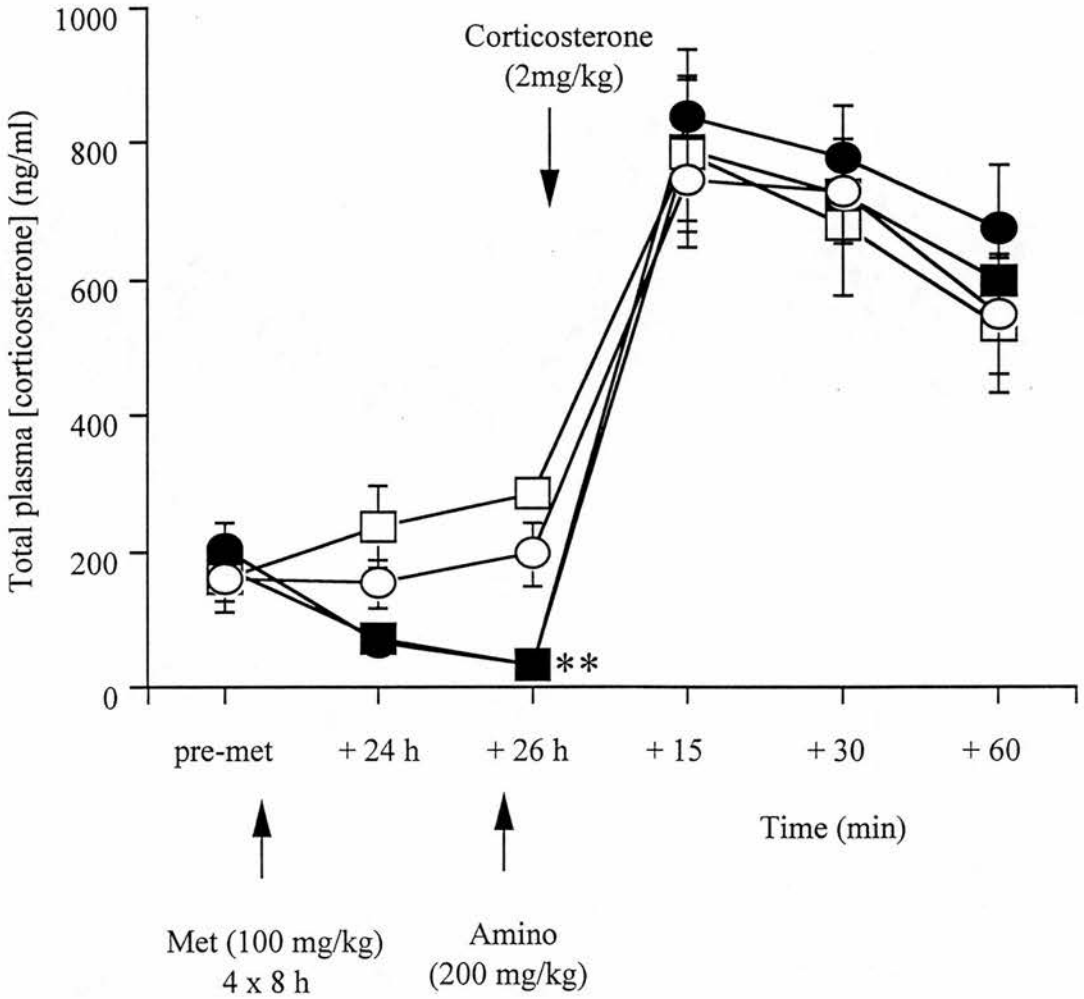


Fig 5.4b: The effect of pharmacological adrenalectomy on the plasma corticosterone levels. A basal blood sample was taken prior to the drug treatment, then the animals were given a subcutaneous (s.c.) injection of either metyrapone (met, 100 mg/kg), the 11β-hydroxylase inhibitor or vehicle (0.9% saline) every 8 h over 24 h. On the morning of the experiment another blood sample was collected followed by the final met injection at 08.00 h. 75 min later a s.c. injection of either aminoglutethimide (amino, 200 mg/kg) , the 20α-hydroxylase inhibitor or vehicle (DMSO) was given then after 45 min later a third basal sample was collected. Immediately afterwards a s.c. injection of corticosterone (2 mg/kg) was given and blood samples collected 15, 30 & 60 min post-injection. Day 21 pregnant: met/amino (■, n = 5) & vehicle (□, n = 3) and virgin: met/amino (●, n = 5) & vehicle (○, n = 5).
Statistical analysis: delta values from pre-met minus + 26 h values analysed by Student's t test, ** $p < 0.01$ vs. pregnant and virgin vehicle groups.

dexamethasone 2-4 days after adx (Reul *et al.*, 1989). This is proposed to be due to enhanced MR mRNA translation and/or the stability of MR receptors by chronic occupation of GR by dexamethasone. Stress also alters hippocampal GRs and MRs with the effects being stress-specific (Holmes *et al.*, 1995; Sapolsky *et al.*, 1994). However, this autoregulation of GR and MR is an acute phenomenon and the effects are not sustained (Herman *et al.*, 1989; Holmes *et al.*, 1995).

In our *in situ* hybridisation studies we did not find a significant difference in GR mRNA expression in either the PVN or the hippocampus and in MR mRNA expression in the hippocampus between pregnant and virgin rats. MR mRNA expression was not measured in the parvocellular PVN neurones primarily because expression is extrememly low and several receptor binding studies have shown a very low density of MR in the PVN (Ruel & de Kloet, 1985) suggesting that MR may not play an important role in transducing the glucocorticoid feedback signal at the level of the PVN.

However, we did find a small but significant increase in GR expression in the dentate gyrus on day 21 of pregnancy compared to day 10 of pregnancy. Previous studies have shown that the regulation of hippocampal GR mRNA is subregion specific and appears to be confined to the dentate gyrus and CA1 subfield (Herman *et al.*, 1989; Holmes *et al.*, 1995). This upregulation may reflect an enhanced influence of central neurotransmitters, such as serotonin, which has been demonstrated to exert potent long-term effects on hippocampal GR and MR expression. Central serotonergic lesions in particular decrease hippocampal GR and MR mRNA expression (Seckl *et al.*, 1990; Yau *et al.*, 1994).

The hippocampus is an extremely complex structure with connections existing between separate cell fields (Amaral & Witter, 1989). This leads to the potential for crosstalk to occur between different cell fields. One report using an anterograde neuronal tracer identified a direct input from the ventral subiculum of the hippocampus to the BNST. Subsequently, the axonal projections from the BNST to the PVN were identified as GABAergic (Cullinan *et al.*, 1993). Both *in vivo* and *in vitro* studies indicate a centrally acting inhibitory role for GABA in the release of CRF and the regulation of the HPA axis. *In vivo*, GABA administered i.c.v prevents surgery-induced activation of the HPA axis (Makara & Stark, 1974) and the release

of CRF into the HPB (Plotsky, 1987). Thus this increased GR mRNA expression in the DG of the hippocampus may indirectly influence the activity of the HPA axis via GABAergic interneurones during late pregnancy. However, the true physiological impact of this small increase in GR mRNA expression in the dentate gyrus is difficult to assess as far as conveying the glucocorticoid feedback signal on the HPA axis is concerned since the hippocampus is not the sole negative feedback site in the brain (for review see Jacobson & Sapolsky, 1991). In addition it should be noted that changes in mRNA levels do not always correlate with changes in receptor binding or function (Chao *et al.*, 1989). Hippocampal MR expression did not significantly differ in any of the subfields or the DG analysed.

We did not detect changes in corticosteroid receptor gene expression in the hippocampus or the PVN during pregnancy, so the suppressed HPA axis responsiveness to an acute stress in the pregnant rat seems unlikely to be due to enhanced feedback sensitivity. Although we have hypothesised that the small increase in GR expression in the dentate gyrus on day 21 of pregnancy may indirectly influence PVN CRF expression via GABAergic interneurones. However, it would be desirable to seek changes in MR and GR receptor binding.

5.4.2 Changes in 11β-HSD1 activity in the PVN and anterior pituitary

In vitro, we found that the 11β-dehydrogenase activity in the PVN doubled between virgin controls and day 16, with an approximately tripling on day 21 of pregnancy. Also the bioactivity in the anterior pituitary increased significantly between day 16 and day 21 of pregnancy. The higher circulating levels of corticosterone that occur during pregnancy (Atkinson & Waddell, 1995, Nolten & Rueckert, 1981) may be responsible for this enhanced activity since the promoter region of the 11β-HSD1 gene possesses a putative glucocorticoid-responsive element (Moisan *et al.*, 1992) and several studies have shown that it is regulated by glucocorticoids. Hippocampal and liver 11β-HSD1 activity and mRNA (Low *et al.*, 1994a; Moisan *et al.*, 1990c) were significantly decreased 10 days after surgical adx, and replacement with dexamethasone resulted in a significant increase (Low *et al.*, 1994a). Similarly animals exposed to chronic stress, in the form of adjuvant-induced arthritis, displayed increased enzyme activity (Low *et al.*, 1994b). Therefore, if this

enhanced *in vitro* activity reflects enhanced dehydrogenase activity *in vivo* this would indicate that during pregnancy in the PVN and to a lesser extent in the anterior pituitary greater inactivation of corticosterone occurs leading to reduced levels of glucocorticoids gaining access to GR and eliciting their feedback effects leading to a reduced sensitivity to the negative feedback signal. However, this is inconsistent with the hyporesponiveness of the HPA axis during stress.

Mounting evidence from primary cell cluture and clonal cell lines suggest that *in vivo* this enzyme may preferentially act as a reductase: reactivating glucocorticoids from their inactive metabolites. COS-7 cells (derived from monkey kidney) transfected with 11β-HSD1 cDNA and primary cultures of rat hepatocytes and fetal hippocampal neurones all exhibited predominantly 11β-reductase activity with little 11β-dehydrogenase activity: converting glucocorticoids to their inactive metabolites (Jamieson *et al.*, 1995; Low *et al.*, 1994a; Rajan *et al.*, 1996). Therefore, we hypothesised that this increased *in vitro* dehydrogenase activity in the PVN during pregnancy may represent increased reductase activity *in vivo*, thus, resulting in an enhanced local production of glucocorticoids in the PVN which may be responsible for the previously demonstrated decrease in PVN CRF mRNA expression during pregnancy (Douglas & Russell., 1994; see Chapter 4).

A previous report found that reductase activity measured in the brain *in vitro* paralleled dehydrogenase activity (Lakshmi *et al.*, 1991). So we examined *in vitro* reductase activity in the anterior pituitary and hippocampus; however, we only detected approximately half and a third, respectively, of the activity measured in the dehydrogenase direction. Therefore, in our assay protocol *in vitro* reductase activity appears to be more unstable than the dehydrogenase activity. This finding is in agreement with other reports (Waddell & Burton, 1993), although one report found that reductase activity measured from brain tissue was more stable than from liver (Lakshmi *et al.*, 1991). It has been proposed that the physicochemical environment of the 11β-HSD1 enzyme is important for reductase stability (Lakshmi & Monder, 1985). One study in the placenta suggested that homogenisation reduces reductase activity by uncoupling the 11β-HSD1 enzyme from other intracellular system(s) (Lopez-Bernal *et al.*, 1982).

5.4.3 The effect of centrally-administered GA on the stress responsiveness of the HPA axis

In order to resolve the *in vivo* role of central 11β-HSD in the modulation of the glucocorticoid negative feedback on the HPA axis responses to stress we administered GA, the active component of liquorice which potently inhibits central 11β-HSD1 (Moisan *et al.*, 1990a), by intracerebroventricular infusion (i.c.v). A previous report by our group demonstrated that following s.c. GA, the hypophysial portal blood levels of CRF decreased at 45 min and 90 min post-injection suggesting that central 11β-HSD may primarily act as a dehydrogenase and thereby regulate the access of corticosterone to GRs expressed in feedback sites, including the PVN (Seckl *et al.*, 1993). However, systemic administration of GA will not necessarily only cause direct inhibition of central 11β-HSD.

In agreement with our own observation (see Chapter 3) and a previous study (Neumann et al., 1998), the maximum plasma ACTH levels following the stress were significantly decreased in the vehicle-treated pregnant group compared to the vehicle-treated virgins. Similarly, the maximum plasma ACTH levels in the GA-treated pregnant were significantly lower compared to the GA-treated virgin controls. However, we demonstrated that central inhibition of 11β-HSD does not have a significant effect on the HPA axis responses to stress. Although at both 5 and 15 min post-stress the plasma ACTH levels of the GA-treated pregnant rats were lower than in the vehicle-treated pregnant group the difference between the two groups was not statistically significant. If basal ACTH concentrations had been measurable we could have calculated the increment between the pre-stress and 5 min post-stress ACTH levels providing an more accurate measurement of the responsiveness of the HPA axis following inhibition of 11β-HSD. This lack of effect of GA on the HPA axis is at odds with the previous report of Seckl *et al* (1993), who found decreased HPB levels of CRF following s.c. administration of GA. This study strongly indicated that GA, through the inhibition of 11β-HSD, was enhancing corticosterone feedback at the PVN and/or extrahypothalamic sites. Interestingly, they also reported an increase in HPB levels of AVP and OXT. Since both of these are known ACTH secretagogues (Antoni et al., 1983a; Giguere & Labrie, 1982) their increased release may simply reflect a compensatory mechanism at the level of the PVN to maintain

ACTH release in the presence of reduced CRF release. This inconsistency may possibly reflect an incorrect GA concentration or an ineffective delivery of the drug. It would be worthwhile to repeat this experiment but rather infuse the GA directly into the PVN.

Interestingly, we found that at 50 min post-stress the plasma corticosterone levels in both pregnant groups were significantly elevated compared to the vehicle-treated virgin group. This is discussed in Chapter 6.

5.4 The sensitivity of the HPA axis to exogenous corticosterone in the pregnant rat

Changes in glucocorticoid feedback during pregnancy appears to be a species dependent phenomenon. Keller-Wood *et al* (1996) found that the sensitivity of hypotension-induced ACTH secretion to inhibition by a cortisol infusion was the same in pregnant and nonpregnant ewes. Owen *et al* (1987) demonstrated that pregnant women are insensitive to feedback inhibition by dexamethasone during pregnancy which persists into the 2nd or 3rd postnatal week. To date no comparable data exist in the rat. We chose pharmacological adx rather than surgical because a previous report demonstrated that surgical adx on day 14 of pregnancy or later did not eliminate the maternal levels of corticosterone and the plasma CBG levels remained unchanged. The explanation is that during late pregnancy in response to surgical adx fetal corticosterone can maintain maternal plasma corticosterone levels and therefore the CBG levels (Cohen *et al.*, 1990). We used a modified method of Plotsky & Sawchenko (1987) with a reduced dose of metyrapone (100 mg/kg) since a dose of 200 mg/kg had previously been reported to cause maternal weight loss and fetal wasting or even death (Baram & Schultz, 1990). Following the metyrapone/aminoglutethimide treatment the plasma corticosterone levels were significantly reduced compared to the vehicle pregnant and virgin control groups ($p < 0.01$) with the levels achieved similar to those previously reported in pregnant rats (Baram & Schultz, 1990). Confirmation of the suppression of the circulating levels of corticosterone was provided by the fact that the plasma ACTH levels were significantly elevated. The maximum concentration of plasma ACTH reached in the virgin and day 21 pregnant animals were similar suggesting that the HPA axis of the

pregnant rat was not under a stronger glucocorticoid influence and that ACTH secretory capacity is similar in pregnant and virgin rats. Following the s.c. administration of corticosterone plasma ACTH levels decreased over time. However, comparison of pre-corticosterone levels with the levels 30 min post-injection revealed that while the plasma ACTH levels in the virgin animals were significantly lower at this time point in the pregnant groups ACTH levels were not significantly reduced. This suggests that pregnant rats are as sensitive to delayed glucocorticoid feedback (manifested between 45 to 60 min after exposure to the corticosteroid) as virgin rats but are less sensitive to fast rate-sensitive feedback (manifested within minutes of exposure to corticosteroid when the plasma levels of steroid are rising). A similar finding was demonstrated in pregnant ewes where basal plasma ACTH levels were less responsive to rapid inhibition by cortisol (Keller-Wood et al., 1996).

In conclusion, we did not find that GR or MR mRNA expression in the PVN and/or hippocampus was significantly increased between pregnant and virgin rats. Although, we did detect a small but significant increase in GR expression in the dentate gyrus across pregnancy, and this may lead to an enhanced inhibition of CRF neurones in the PVN via GABAergic interneurones.

The enhanced 11β-HSD1 bioactivity in the PVN and to a lesser extent in the anterior pituitary, measured *in vitro*, may *in vivo* reflect increased reductase activity resulting in a greater local production of corticosterone; this may explain the previously seen decrease in parvocellular CRF mRNA expression during pregnancy. However, the effects of *in vivo* inhibition of central 11β-HSD by GA tentatively suggests that 11β-HSD may act predominantly as a dehydrogenase, inactivating glucocorticoids in brain regions associated with the regulation of the HPA axis, including the PVN.

Finally, the HPA axis of the pregnant rat is as sensitive to delayed negative feedback, or adx as in the virgin rat but is less responsive to rapid inhibition by corticosterone which does not explain the reduced HPA axis responsiveness to stressors in pregnancy. Thus the attenuated HPA axis response to acute stress in late pregnancy is not due to an enhanced glucocorticoid feedback signal, rather to a change in the forward drive to the HPA axis at the level of the PVN and anterior pituitary (see Chapter 4).

CHAPTER 6

The influence of central endogenous opioid systems on the HPA axis during pregnancy

6.1 Introduction

Endogenous opioid peptides are widely distributed in the CNS and they are derived from several large precursor molecules; proopiomelanocortin (POMC) gives rise to several biologically active peptides, including ACTH, however; it also is the precursor for β-endorphin; Proenkephalin A is the precursor for several opioid peptides, including lue-enkephalin, met-enkephalin, met-enkephalin-arg-phe, met-enkephalin-arg-gly-leu. Prodynorphin also contains several opiate active peptides, including dynorphin A, dynorphin B (rimorphin) and α- and β-neoendorphin (for review see Khachaturian *et al.*, 1985).

The ability of endogenous opioid peptides (EOPs) and opiate drugs, such as morphine, to influence the HPA axis has been known for more than half a century (Selye, 1936). While studies performed in humans support a clear inhibitory role of endogenous opioids (Grossman *et al.*, 1986; Volavka *et al*, 1980), studies in rats have demonstrated both inhibition (Slusher & Browning, 1961; Buckingham & Cooper, 1986) and a facilitation (De Souza & Van Loon, 1982; Buckingham & Cooper, 1986).

A number of *in vivo* and *in vitro* studies have tried to address the issues of whether the influences of EOPs on the HPA axis are primarily inhibitory or stimulatory; the location of their site(s) of action, such as on the corticotrophs in the anterior pituitary or on the nerve terminals in the external zone of the median eminence or on parvocellular CRF cell bodies in the PVN or on extrahypothalamic nerve terminals in the PVN; and the specific opioid receptor subtypes involved.

Morphine, a naturally-occuring alkaloid from the poppy, *Papaver somniferum* and etorphine, a synthetic μ-opiates, *in vivo* cause a dose-dependent increase in

plasma corticosterone (Iyengar *et al.*, 1986) and *in vitro* FK33-824CH and Try-*D*-Ala-Gly-MePhe-NH$(CH_2)_2$OH, specific μ-opioid receptor agonists, increase the secretion of immunoreactive CRF (I-CRF) from isolated rat hypothalami (Buckingham, 1982b; Buckingham & Cooper, 1986). Studies using the endogenous opioid peptide, β-endorphin, derived from the POMC precursor, have shown mixed effects. *In vitro*, β-endorphin treatment inhibits both spontaneous and stimulated secretion of CRF from isolated hypothalami (Tsagarakis *et al.*, 1990). In contrast, Buckingham (1986) demonstrated that this effect was dose-dependent. Exposing hypothalamic explants to β-endorphin over a concentration range of 10^{-7}-10^{-5} M inhibited bioactive CRF (B-CRF) release while exposure to much lower concentrations (10^{-11}-10^{-10} M) stimulated release. *In vivo*, however, β-endorphin appears to have primarily an inhibitory effect on the HPA axis since i.c.v administration decreases the spontaneous and nitroprusside hypotension-induced release of I-CRF into the hypophysial portal system in anaesthetised rats (Plotsky, 1986) and the hypoglycaemia-induced elevations in hypothalamic CRF and anterior pituitary POMC mRNA expression and plasma ACTH concentrations (Suda *et al.*, 1992). However, one report has shown that β-endorphin i.c.v. causes an elevation of plasma corticosterone levels (Iyengar *et al.*,1987). The consensus is that β-endorphin fulfils a role as an endogenous ligand of the μ-opioid receptor. However, in receptor binding studies it has been shown to possess multiple opioid receptor affinities (Wood *et al.*, 1981). In animals made tolerant to morphine, U50 488H, a selective κ-opioid receptor agonist, or morphine and D-Ala^2-D-Leu^5-enkephalin (DADLE), a specific δ-opioid receptor agonist, the effect of β-endorphin is only partially inhibited (Iyengar *et al.*, 1987) indicating that *in vivo* it posseses the ability to act at all the opioid-receptor subtypes or might act at an additional receptor site. Some studies suggest the existence of a unique β-endorphin or ε-opioid receptor (Law *et al.*, 1979; Schulz *et al.*, 1980), however, very little is known about this opioid receptor subtype.

One study investigating the influence of the σ-opioid receptor on corticosterone secretion *in vivo* reported that the potent σ-opioid receptor agonists, D-cyclazocine and D-ethylketocyclazocine, did not increase plasma corticosterone levels (Iyengar *et*

al.,1986), therefore, indicating that the σ-opioid receptor subtype is not involved in the regulation of the HPA axis.

The EOPs, met- and leu-enkephalins, derived from the proenkephalin A precursor, have previously been shown to increase the CRF content in and secretion from the hypothalamus when administered i.p. (Buckingham, 1982), however, a subsequent study by the same group demonstrated that isolated rat hypothalami incubated with D-Pen^2-D-Pen^5-enkephalin, a δ-opioid receptor agonist, did not respond, indicating that δ-opioid receptors are not involved in the initiation of the secretion of the releasing hormone (Buckingham & Copper, 1986). Two subsequent *in vivo* studies, however, clearly demonstrated that the δ-opiates potently and dose-dependently increased plasma ACTH and corticosterone (Gonzalvez *et al.*, 1991; Iyengar *et al.*, 1986) thus providing evidence of a δ-opioid input site into the HPA axis.

An initial *in vitro* study found that the κ-opioid receptor agonist, U50 488H, was only weakly active at stimulating the release of CRF from isolated hypothalami (Buckingham & Cooper, 1986) while other studies provided evidence that the κ-opioid receptor plays an inhibitory role on the function of the HPA axis (Plotsky, 1986; Tsagarakis *et al.*, 1990). However, later *in vivo* studies clearly demonstrated that κ-opioid peptides and their receptors were involved in the regulation of the HPA axis. Iyengar and colleagues (1986 & 1987) showed that synthetic opiates, U50 488H, MR2034 and trifluadom, and κ-opioid peptides, dynorphin and met-enkephalin-arg-phe, a C-terminally extended met-enkephalin which *in vitro* possesses a high affinity for κ-opioid receptors (Audigier et al., 1982), dose-dependently and stereospecifically increased plasma corticosterone. Interestingly, Iyenger *et al* (1986) found that the effects of trifluadom but not of U50 488H were reversed by the opioid antagonist, WIN 44441-3 thus providing the first *in vivo* evidence for multiple κ-isoreceptors. Immunoneutralization of hypothalamic CRF by a specific antiserum to rat CRF (CRF-AS) (Nikolarakis *et al.*, 1987) resulted in a complete abolition of the ACTH response to MR2034, indicating that κ-opioid receptor regulation of the HPA axis occurred at the hypothalamus or at extrahypothalamic nerve terminals in the PVN.

The role of the anterior pituitary gland in the opioid regulation of the HPA axis was first investigated by Buckingham (1982) who found that neither morphine nor met- and leu-enkephalin stimulated ACTH secretion from anterior pituitary quarters and concluded that the anterior pituitary did not express either μ- and δ-opioid receptors and therefore was not a regulation site for opioids. A subsequent study, however, demonstrated that MR2034 stimulated the release of ACTH from primary cultured anterior pituitary cells (Calogero *et al.*, 1996) despite the paucity of κ-opioid receptors in this lobe (Boersma *et al.*, 1994). This influence of κ-opioid receptor agonists at the level of the anterior pituitary helps to explain why the stimulating effects of MR2034 on plasma ACTH and corticosterone were not completely abolished in the presence of the CRF receptor antagonist, α-helical CRF_{9-41}. However, stimulation at the level of the hypothalamus of other ACTH secretagogues such as AVP and noradrenaline may occur. κ-opioid peptides do not directly influence the adrenal gland, since MR2034 had no effect on the release of corticosterone from cultured adrenocortical cells or adrenal quarters *in vitro* (Calogero *et al.*, 1996).

Thus, μ-, κ-, δ- and possibly ε-opioid receptors are involved in the regulation of the HPA axis and EOPs appear to act at the level of the hypothalamus and possibly on inputs from extrahypothalamic regions in male rats, and possibly on the anterior pituitary.

An example of the influences of central EOPs on a neuroendocrine system occurs in the hypothalamic-neurohypophyseal system (HNS) during pregnancy. EOPs provide an inhibitory tone on both the magnocellular oxytocin neurones of the SON and on the nerve terminals in the neurohypophysis (Leng *et al.*, 1985) to regulate the release of oxytocin. The oxytocin content of the posterior pituitary is increased during pregnancy (Douglas *et al.*, 1993; Schriefer, 1991) and this excess store is secreted at parturition to stimulate uterine contractility and promote fetal expulsion. Some studies have found no change in the expression of hypothalamic oxytocin mRNA during pregnancy (Douglas & Russell, 1994) suggesting that this accumulation, at least in part, may be due to a restraint on the secretion via EOPs.

However, there have been other reports of enhanced expression, so this remains controversial (Horwitz *et al.*, 1994).

The endogenous opioid peptides exert their inhibitory tone by decreasing the electrical activity of the oxytocin cell bodies in the magnocellular hypothalamus. The EOP thought to be responsible is β-endorphin since the effect appears to be mediated via μ-opioid receptors located pre-synaptically on the noradrenergic nerve terminals in the SON, arising from the A2 brainstem group, which is stimulated by uterine cervical distension (Onaka *et al.*, 1995). EOPs also act directly on the neurosecretory terminals in the neurohypophysis to prevent the release of oxytocin. This effect is proposed to be mediated possibly by dynorphin or more likely by C-terminally extended enkephalin peptides acting via κ-opioid receptors (Clarke et al., 1990), since proenkephalin-derived peptides, and to a lesser extent dynorphin, have been found to be colocalised with oxytocin in the posterior pituitary (Panula & Lindberg, 1987). Towards the end of pregnancy there is a downregulation of the κ-opioid receptors in the posterior pituitary (Sumner *et al.*, 1992) which may reflect an excess exposure to EOPs. However, the influence of the κ-opioid peptides on the neurosecretory nerve terminals in the posterior pituitary is proposed to be important in initially building up the oxytocin stores. The physiological role of this increased opioid inhibitory tone on oxytocin secretion during pregnancy is likely to be important in controlling the speed of parturition.

In this study we were interested to see whether central endogenous opioid systems were involved in the attenuation of the HPA axis during pregnancy since it is well documented that the levels of the central EOPs change during pregnancy. For example, the hypothalamic content of β-endorphin increases (Wardlaw & Frantz, 1983); dynorphin mRNA levels in the supraoptic nucleus and content in the neurohypophysis are also enhanced; and although enkephalin expression in the neurohypophysis increases (Schriefer, 1991), a recent study was unable to detect any change in the expression of proenkephalin A mRNA in the PVN in late pregnancy (Douglas & Russell, 1994).

6.2 Materials and Methods

Virgin female Sprague Dawley rats were individually housed with sexually experienced males and mated overnight. Pregnancy was determined by the presence of a vaginal plug of semen and referred to as day 0 of pregnancy.

See Chapter 2 for details. Briefly, the individually caged pregnant and virgin control animals each had a silastic cannula inserted in a jugular vein by the method described in detail in and were allowed to recover for three to four days prior to experimentation.

On the morning of the experiment, sampling cannulae attached to 1 ml syringes filled with sterile heparinised saline (20 IU/ml 0.9% saline) were connected between 07.00 and 08.00 h and the animals left undisturbed for 90 min. Two basal blood samples were taken at 09.30 and 10.00 h. A volume of 0.65 ml was taken at both times and immediately substituted by sterile 0.9% saline. Then naloxone (5 mg/kg) or vehicle (sterile 0.9% saline) was injected intravenously (i.v.) and blood samples taken 5 and 15 min post-injection. The rats were then exposed to forced swim (FS) stress (90 s): animals placed in a bucket filled with tap water (19 ^{o}C, depth of 40 cm). After the swim, they were gently towel dried for 10 s and returned to their home cages where further blood samples were taken at 5, 15, 30 and 60 min post-stress. At the end of the experiment the animals were killed by an i.v. overdose of anaesthetic (Sagatal,) and their pregnancy status checked.

All blood samples were collected into EDTA coated tubes supplemented with aprotinin (0.039 TIU/tube, Sigma, UK), a protease inhibitor, on ice and centrifuged for 5 min. The plasma was aliquoted (80 µl for ACTH, 50 µl for corticosterone and 30 ml for corticosterone binding globulin, CBG) and stored at -80 ^{o}C and -20 ^{o}C, respectively, until assay. The plasma ACTH levels were determined by a commercially available kit (ICN, USA) by Prof. R. Landgraf's group in Munich, Germany. Plasma corticosterone and CBG levels were determined by specific and sensitive assays. For details see Chapter 2.

6.3 Results

6.3.1 The effect of naloxone (nlx) on the HPA axis secretory responses to FS stress

Two way ANOVA for repeated measures (RM) of the plasma ACTH and corticosterone concentrations in both groups demonstrated a significant interaction between time and group ($p < 0.001$); all other specific comparisons were derived from Newman-Keuls t test ($p < 0.05$) unless otherwise stated.

There was no difference in the basal levels of ACTH between virgin and day 21 pregnant animals and nlx did not significantly affect basal ACTH within 15 min of administration in either the virgin or pregnant group (fig 6.1a). FS stress significantly increased ACTH secretion in both vehicle- and nlx-treated virgin and pregnant rats, compared to pre-swim levels ($p < 0.05$), reaching a maximum 5 min post-stress. The ACTH secretory response to forced swim was significantly lower in vehicle-treated pregnant rats compared to vehicle-treated virgin controls ($p < 0.05$). Nlx significantly attenuated the ACTH secretory response in virgins ($p < 0.05$) and caused a small, but non significant increase in the secretory response in pregnant rats which resulted in no difference between nlx-treated virgin and pregnant rats after stress. Further analysis showed that the effect of nlx on the ACTH secretory response to stress was significantly different between the virgin and pregnant groups (t-test on the differences between the plasma ACTH level after FS stress in nlx- and vehicle-treated groups and the calculated s.e.m. of the difference of the two independent means, $p < 0.05$, virgin -103 ± 57 vs. pregnant $+40 \pm 29$ pg/ml). At 15 min post-stress there was no difference in plasma ACTH levels among groups. Plasma ACTH levels in both virgin groups 60 min after the end of the stress were significantly lower than their respective maximum responses while the plasma level in the vehicle-treated pregnant group was not significantly reduced; however, this is probably a consequence of the lower maximum ACTH response achieved in the latter group. In

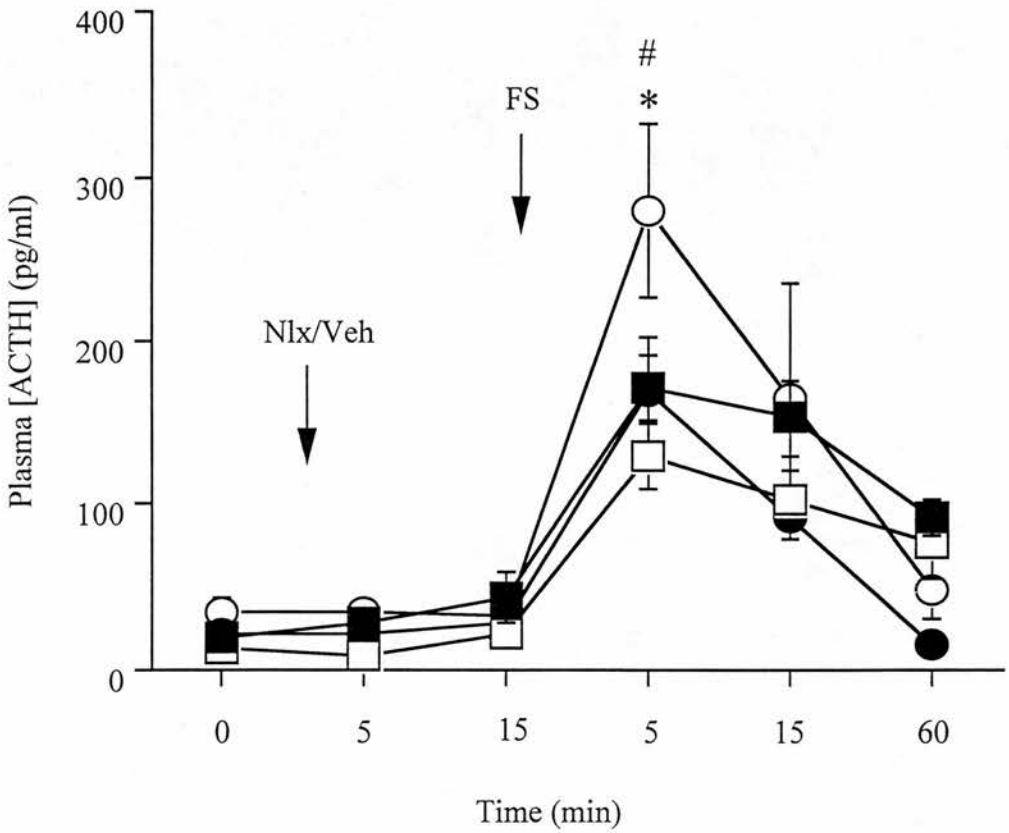


Fig 6.1a: Effect of naloxone (nlx) on the ACTH secretory response to forced swim (FS) stress in pregnancy. Groups are: virgin vehicle-treated (○) & nlx-treated (●) and day 21 pregnant vehicle-treated (□) & nlx-treated (■). Two basal blood samples were collected. These were not significantly different so were pooled together as a single basal sample. Nlx (5 mg/kg) or vehicle (sterile 0.9% saline) were given i.v. 30 min later and two samples collected 5 and 15 min post-injection. Immediately afterwards, each rat was exposed to FS stress for 90 s and samples were collected 5, 15 & 60 min post-stress. Data are expressed as group means ± S.E.M. Two way ANOVA for RM: $p < 0.0001$ followed by Newmann Keuls test; * $p < 0.05$ vs. pre-swim levels, # $p < 0.05$ vehicle-treated virgin vs. all other groups at 5 min post-stress.

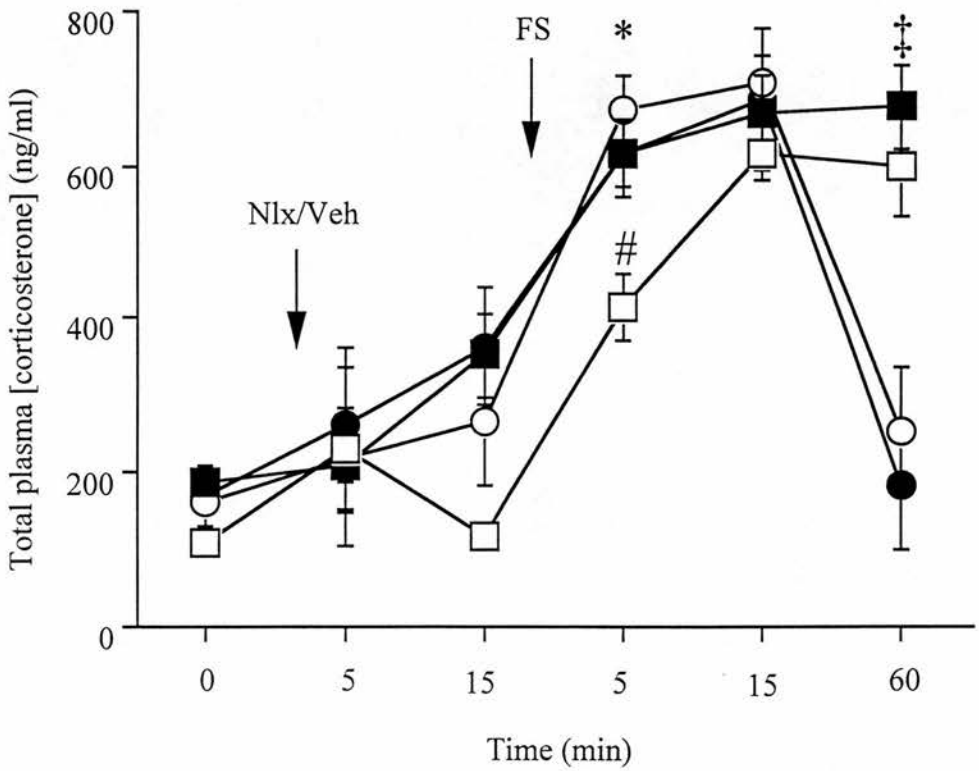


Fig 6.1b: Effect of naloxone (nlx) on the corticosterone secretory response to forced swim (FS) stress in pregnancy. Virgin vehicle-treated (○) & nlx-treated (●) and day 21 pregnant vehicle-treated (□) & nlx-treated (■). Two basal blood samples were collected; however, these were not significantly different so were pooled together as a single basal sample (time 0). Nlx (5 mg/kg) or vehicle (sterile 0.9% saline) were given i.v. 30 min later and two samples collected 5 and 15 min post-injection. Immediately afterwards, each rat was exposed to FS stress for 90 s and samples were collected 5, 15 & 60 min post-stress. Data was expressed as group means ± S.E.M. Two way ANOVA for RM followed by Newmann Keuls test: * $p < 0.05$ vs. pre-swim levels (time0), # $p < 0.05$ vs. vehicle-treated virgin & ‡ $p < 0.05$ pregnant vs. virgin.

addition, at this time point there was no difference in plasma levels among the groups.

Similarly the basal corticosterone concentrations did not differ between virgin and pregnant rats and nlx did not affect plasma levels within 15 min of administration in either the virgin or pregnant group (fig 6.1b). FS stress significantly increased corticosterone secretion in all groups compared to pre-swim levels ($p < 0.05$) with the corticosterone secretory response at 5 min post-stress being significantly less in vehicle-treated pregnant compared to vehicle-treated virgin controls ($p < 0.05$). Nlx did not affect the secretory response in virgin animals 5 min after the end of the stress; however, it did significantly increase the response in the pregnant rats ($p < 0.05$). Therefore, as with ACTH, the effect of nlx on the stress response was significantly different between virgin and pregnant rats (t-test on the difference between the plasma corticosterone level after FS stress in nlx- and vehicle-treated groups and the calculated independent means, $p < 0.05$: virgin, - 60.3 ± 66.4 vs. pregnant +199.5 ± 62.3 ng/ml). The maximum corticosterone levels occurred 15 min post-stress with no difference among groups. Consistent with the plasma ACTH levels, the plasma corticosterone concentrations 60 min after the end of the stress in both pregnant groups remained elevated compared to virgin controls and were not significantly reduced compared to their respective maximum responses.

6.3.2 Comparison of basal plasma CBG levels between pregnant and virgin rats

The basal levels of plasma CBG did not significantly differ between virgin and pregnant rats (14.8 ± 3.6 vs. 16.2 ± 4.7 pmol [^{3}H]corticosterone bound/mg of protein, respectively (fig 6.2).

6.4 Discussion

In this study we revealed that central EOPs are involved in the stimulation of ACTH secretory response during acute stress in female virgin rats and that during pregnancy this endogenous opioid enhancing effect appears to be lost.

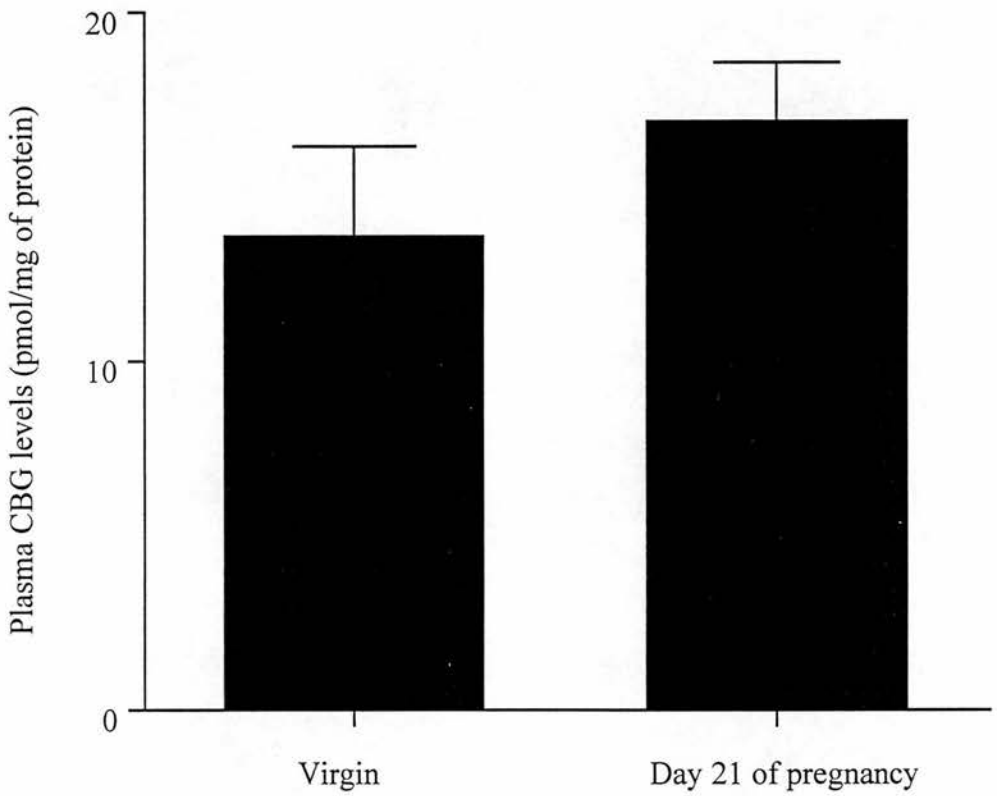


Fig 6.2: Plasma levels of corticosterone binding globulin (CBG) from virgin (n = 5) and day 21 pregnant (n = 5) rats. Measurements were made in basal blood samples collected prior to either naloxone/vehicle injection or exposure to forced swim (FS) stress. Data are expressed as group means ± S.E.M. Student's t test: no significant difference.

Nlx did not affect the basal secretion of ACTH or corticosterone in either virgin or pregnant groups. Thus, there appears to be no endogenous opioid tone on the basal activity of the HPA axis. This finding is in agreement with one study which reported that a single injection of 5 mg/kg naloxone did not affect plasma corticosterone concentrations, although when two injections were given consecutively a significant reduction was seen (Iyengar *et al.*, 1986) and may therefore be a concentration-dependent effect. However, several other studies have shown that administration of either naloxone or naltrexone, another opiate antagonist, can stimulate both CRF and ACTH secretion (Eisenberg, 1980; Nikolarakis *et al.*, 1987; Plotsky, 1986). The difference between those studies and ours may reflect the time period between administration of nlx and the blood sampling. However, electrophysiological studies investigating the effects of nlx on the electrical activity of oxytocin neurones have shown that the maximum effect following systemic administration occurred 5 to 10 min post-injection (Bicknell *et al.*, 1988). Therefore, in our study we would have expected to see an effect of nlx at the sampling times chosen. Alternatively, the difference may reflect differences in routes of administration, in our study nlx was given i.v while in the other studies it was given either i.c.v (Plotsky et al., 1986) or i.p. (Iyengar et al., 1986).

Virgin rats responded to FS stress with a robust increase in ACTH secretion in both vehicle- and nlx-treated groups, however, the secretory response of the nlx-treated animals was significantly reduced compared to its controls ($p < 0.05$). This indicates that *in vivo* in response to an acute stress, such as FS stress, central EOPs are involved in the stimulation of the HPA axis. This result is supported by several *in vivo* studies (Buckingham, 1986; Iyengar *et al.*, 1986, 1987; Gonzalvez *et al.*,1991; Calogero *et al.*, 1996). Our study does not, however, enable us to determine which opioid receptor subtypes are involved since the dose of nlx used was not only able to antagonise the nlx-sensitive μ-opioid receptor but was of a high enough concentration to affect the more nlx-resistant δ- and κ-opioid receptors (Howlett & Rees, 1986). Therefore, use of selective antagonists would be required to specifically elucidate the receptors involved. A number of studies have reported that μ-, δ-, κ-opioid receptors at the level of the hypothalamus and possibly in the anterior

pituitary are involved in the modulation of the HPA axis (Iyengar *et al.*, 1987; Calogero *et al.*, 1996). Indeed, high concentrations of all opioids and their receptors are present in the hypothalamus and median eminence: β-endorphinergic neurones originating in the arcuate nucleus have been identified as projecting to the median eminence and neurones containing POMC-derived peptides appear to send fibres to the PVN (Kiss *et al.*, 1984a; Sawchenko *et al.*, 1982); several hypothalamic nuclei contain high concentrations of enkephalinergic neurones (Cuello, 1983) and although it has previously been shown that the hypothalamus is almost devoid of δ-opioid receptors proenkephalin mRNA has been colocalised to CRF neurones in the parvocellular PVN and its expression appears to be responsive to acute stress (Lightman & Young III, 1987); prodynorphin opioids are largely localised in the magnocellular neurones of the SON and PVN which project to the posterior pituitary, however, dynorphin has been colocalised with CRH and is believed to be co-secreted into the hypophysial portal circulation (Roth *et al.*, 1983). Therefore, the EOPs present in the hypothalamus and pituitary may regulate the HPA axis via autocrine and ultrashort positive feedback mechanisms in the virgin female rat.

The influence of nlx on the corticosterone secretory response in virgin rats was not as clear as with the ACTH response, so although the level at 5 min post-stress was lower in the nlx-treated group it was not significantly different compared to vehicle-treated controls. In addition, the peak corticosterone response occurred at 15 min post-stress, which agrees with previous reports (Neumann *et al.*, 1998). This may reflect the fact that peak corticosterone responses are at or near maximal levels even at low stress intensities (Keller-Wood & Dallman, 1984) therefore plasma ACTH measurements reflect more accurately what is occurring at the level of the hypothalamus.

The ACTH secretory response to stress of the vehicle-treated pregnant rats was significantly reduced compared to their virgin controls which is consistent with our previous results (see Chapters 3 & 5) and Neumann *et al* (1998). Nlx-treatment caused a small elevation in the ACTH secretory response, although it was not significantly different; this results in there being no difference in the stress response between virgin and pregnant nlx-treated rats, although this slight increase makes the

effect of nlx on the stress response significantly different between virgin (ACTH decreased) and pregnant rats.

Therefore, during late pregnancy there is a loss of the stimulatory effect of EOPs on the HPA axis during acute stress. This might be due to opioid receptor downregulation or uncoupling of receptors from their second messenger system, perhaps as a consequence of the increased hypothalamic EOP content during pregnancy (Wardlaw & Frantz, 1983). Alternatively, it may reflect an inhibitory opioid tone being substituted for a stimulatory one.

The pattern of the corticosterone secretory response in the vehicle-treated pregnant group in general followed that of ACTH, with the secretion after stress being significantly reduced compared to the vehicle-treated virgin controls (Neumann et al., 1998).

The plasma level of corticosterone remained significantly elevated for longer in nlx- and vehicle-treated pregnant groups compared to their virgin controls. This prolonged secretion, also seen in our other studies, may represent fetal contribution of corticosterone (Cohen et al., 1990) since by day 21 of pregnancy the fetal HPA axis is independently active (Dupont *et al.*, 1991) and responsive to stress in the mother (Robinson *et al.*, 1988). Alternatively, the high circulating levels of estrogen occuring during pregnancy may play a role by directly stimulating the adrenal gland to secrete corticosterone (Holzbauer, 1957). In addition , the metabolic clearance rate of corticosterone may be reduced during pregnancy due to increased circulating levels of CBG (Seal & Doe, 1967). However, when we measured plasma CBG we found the levels although slightly higher in the pregnant animals were not significantly different compared to the virgin rats. This is in constrast to previous studies where CBG levels have been shown to increase during pregnancy (Rosenthal *et al.*, 1969). The difference in findings may reflect techinical differences in the binding assays used. However, a report by Waddell & Atkinson (1994), found that the metabolic clearance rate of corticosterone was unaffected during pregnancy due to the presence of the placenta and the extra transuterine extraction.

In conclusion, EOPs have a stimulatory effect on the HPA axis responses to acute stress in virgin female rats while in late pregnant rats the HPA axis is no longer

stimulated by opioid peptides and may possibly be under an inhibitory tone. This may reflect an adaptation, through an as yet unknown mechanism, contributing to the suppression of the HPA axis responses to acute stress in late pregnancy.

Chapter 7

The influence of ovarian hormones on the HPA axis during pregnancy

7.1 Introduction

A close association exists between the HPA axis and the hypothalamic-pituitary-ovarian (HPO) axis. This interaction is clearly evident during aberrant functioning of either axis, for example the absence of a normal adrenal diurnal rhythm results in irregular ovarian cycles in rodents (Ramaley, 1975), while abnormal ovarian steroidogenesis produces abnormal regulation in the HPA axis (Biller *et al.*, 1990).

In general, the HPA axis exerts inhibitory effects on the HPO axis, with CRF and POMC-derived peptides, such as β-endorphin, inhibiting hypothalamic gonadotropin-releasing hormone secretion and glucocorticoids inhibiting pituitary luteinizing hormone and ovarian estrogen and progesterone secretion and rendering estrogen-target tissues resistant to the ovarian steroid. These effects are responsible for the hypothalamic amenorrhoea of stress (Laatikainen, 1991) and the hypogonadism that occurs in Cushing's syndrome.

In contrast, the majority of existing data support an activational role of ovarian hormones, especially estradiol, in HPA axis regulation. As far back as the early 1960s it was demonstrated that the activity of the HPA axis exhibited a sexual dimorphism. The pituitary ACTH content and plasma corticosterone concentrations under basal conditions and the ACTH and corticosterone secretory responses to stress are higher in females than in males (Critchlow *et al.*, 1961; Kitay, 1963). Several studies looking at whether basal plasma ACTH and corticosterone levels paralleled the fluctuations in plasma ovarian hormone levels during the estrous cycle found that both plasma ACTH and corticosterone concentrations were higher during late proestrus, compared to all other days of the cycle (Butcher *et al.*, 1974; Carey *et al.*, 1995). In addition, some studies have shown that stress-induced elevations in plasma

ACTH and corticosterone levels are greater in animals in proestrus (Carey *et al.*, 1995; Viau & Meaney, 1991). Finally the hypothalamic PVN CRF mRNA content and activity have been shown to increase on the afternoon of proestrus (Bohler *et al.*, 1990; Hiroshige & Wafa-Okada, 1973). All these effects coincide with the maximal plasma levels of estradiol and progesterone. Thus, there is strong evidence *in vivo* that the ovarian steroids have a regulatory influence on the HPA axis.

Direct evidence for a role of estradiol in the regulation of the HPA axis has been provided by several studies. It has been demonstrated that administration of exogenous estrogen increases the secretion of corticosterone from the adrenal gland (Holzbauer, 1957) and that ovariectomy (OVX) causes the adrenal corticosterone concentration to decrease. In addition estradiol has been shown to influence the HPA axis at various levels. Exogenous estradiol, independently of ACTH, increases the secretion of corticosterone from adrenal gland homogenates of ovariectomized rats (Kitay *et al.*, 1965) and also affects both the synthesis and release of ACTH from the anterior pituitary (Kitay, 1963). However, a later report was unable to induce the release of ACTH from the anterior pituitary by estradiol (Buckingham, 1982a). This discrepancy may be due to differences in the length of time the tissues were exposed to the steroid. Estradiol also stimulates the CRF gene directly via a specific estrogen-response element located in the promoter region of the human gene (Vamvakopoulos & Chrousos, 1993).

In vivo OVX decreases pituitary synthesis and release of ACTH and adrenal synthesis of corticosterone (Coyne & Kitay, 1969; Kitay, 1963) which can be reversed with estradiol treatment (Kitay, 1963). In OVX steroid replaced animals basal and stress-induced plasma ACTH and corticosterone are enhanced in estradiol- and estradiol plus progesterone-treated animals compared to OVX controls (Burgess & Handa, 1992; Carey *et al.*, 1995; Viau & Meaney, 1991).

In addition to estradiol stimulating the HPA axis via effects on CRF, ACTH and corticosterone synthesis and release it may also influence the glucocorticoid negative feedback mechanism mediated via GR and MR. Sex differences in MR and GR binding parameters and levels of transcription have been reported. Chronic estradiol treatment results in the loss of the ability of glucocorticoids to autoregulate

GR and decreases GR binding and transcript levels in anterior pituitary, hypothalamus and hippocampus (Burgess & Handa, 1992, 1993; Pfieffer & Barden, 1987). Effects of this steroid have also been described for MR. Carey *et al*, (1995) found a decrease in both MR binding and transcript levels in the hippocampus following estradiol-treatment in OVX rats which is in agreement with a report by Burgess and Handa, (1993) who found that estradiol treatment induced a decrease in MR mRNA expression in the hypothalamus and hippocampus. These changes indicate a decrease in the potency of the glucocorticoid negative feedback signal, thus resulting in a prolonged stress response (Burgess & Handa, 1992; Viau & Meaney, 1991).

In contrast, there are reports of inhibitory effects of estradiol on the HPA axis. Some studies have found following chronic administration of estradiol that the CRF releasing activity of median eminence extract (Kitay, 1963) and hypothalamic CRF content are decreased (Haas & George, 1989).

However, the mass of evidence points towards a stimulatory effect of estradiol on the HPA axis.

The influence of progesterone on the HPA axis is less well-defined than that of estradiol. Progesterone is known to bind to both GR and MR and in intact cells it acts as an antiglucocorticoid by binding to GR and preventing its nuclear translocation and thus activity (Kaiser *et al*., 1974; Rosseau *et al*., 1978). Infusion of low doses of progesterone in nonpregnant ewes resulted in an antagonism of the cortisol-induced inhibition of ACTH secretion (Keller-Wood *et al*., 1988). Although at higher concentration it can exhibit partial agonistic activity. *In vitro*, progesterone has been shown to decrease CRF-induced ACTH secretion from cultured pituitaries and to inhibit hypothalamic CRF release (Buckingham, 1982a). In OVX rats, replaced with estradiol plus progesterone, the ACTH and corticosterone responses during stress are lower than those of the estradiol-treated animals suggesting that progesterone inhibits the facilitatory effects of estradiol on the HPA axis (Viau & Meaney, 1991). However, a subsequent study by Carey and colleagues (1995) did not support a definite inhibitory role for progesterone since they found it did not affect either basal or stimulated HPA axis activity although they did find that it attenuated the estradiol-

induced decrease in MR binding capacity by reversing the estradiol -induced decrease in MR mRNA expression in the hippocampus. This antagonism by progesterone of estradiol-induced effects via its progesterone receptor is well documented. However, in the same study cytosol binding analysis revealed that progesterone in the presence or absence of estrogen priming decreased the apparent binding affinity of MR. This may reflect occupation of MR by progesterone or one of its metabolites, such as 11β-hydroxyprogesteone which is likely to be inhibitory (Rupprecht *et al.*, 1993). Interestingly, progesterone has previously been shown to lower the affinity of the GR complex by increasing the rate of dissociation of glucocorticoids from its receptor (Svec *et al.*, 1980). Thus, the influence of progesterone on the HPA axis appears quite complex and is likely to involve an interaction with estradiol.

Thus, the ovarian steroids modulate the activity of the HPA axis. To date all the reports examining the interaction between the HPA and HPO axes have concentrated on the menstrual and estrous cycles or in OVX animals replaced with ovarian steroids and not during pregnancy when plasma levels of estrogens and progestogens are extremely high. Therefore in this study we wanted to see whether these high circulating levels of ovarian steroids replicated in nonpregnant rats would influence the response of the HPA axis to acute stress. In a separate study we examined whether the dramatic fall in the plasma progesterone levels which occurs during the last two days of pregnancy may be involved in the hyporesponsiveness of the HPA axis to acute stress.

7.2 Materials and Methods

For details see Chapter 2. Briefly, in the first study intact female virgin Sprague Dawley rats (230-250 g) were implanted with a s.c. estradiol- (15 mg/ml) or vehicle-containing (89.7% arachis oil, 10% benzyl alcohol & 0.3% cresol) silastic capsule followed two days later with seven progesterone-containing or vehicle (empty) capsules. Two weeks later jugular cannulae were fitted under halothane: nitrous oxide anaesthesia to allow for blood sampling. At the time of this surgery a blood sample was collected for the measurement of plasma estradiol and progesterone

concentrations. The animals were then allowed to recover for three days prior to experimentation.

On the morning of the experiment between 07.00 and 08.00 h sampling cannulae fitted with 1 ml plastic syringes, filled with heparinised sterile saline (20IU/ml 0.9% sterile saline), were connected. Two basal blood samples were taken at 9.30 and 10.00 h and then each animal was exposed to 90 sec of forced swim (FS) stress. Following the stress the animals were gently towel-dried for 10 sec and then returned to their home cages. Subsequent blood samples were collected 5, 15, 25 and 50 min after the end of the stress. A volume of 0.25 ml of blood was collected at each time point and immediately replaced with an equal volume of 0.9% sterile saline.

At the end of the experiment a blood sample (0.8 ml) was collected for ovarian steroid plasma determinations then the animals were killed by i.v. overdose of anaesthetic (Sagatal, 600 μl/animal).

In a second experiment the same ovarian steroid s.c. implantation protocol as above was used, except that during the jugular cannulation surgery the seven progesterone capsules were removed from controls to simulate the fall in plasma progesterone levels that occurs towards the end of pregnancy. The animals were then left to recover for two days prior to experimentation.

On the morning of the experiment between 07.00 and 08.00 h sampling cannulae fitted with 1 ml plastic syringes, filled with heparinised sterile saline (20U/ml 0.9% sterile saline), were connected. Two basal blood samples were taken at 9.30 and 10.00 h and then each animal was exposed to 90 sec of forced swim (FS) stress. Following the stress the animals were gently towel-dried for 10 sec and then returned to their home cages. A final blood sample was collected 5 min after the end of the swim. Following a blood sample an equal volume of 0.9% sterile saline was infused back into the animal. At the end of the experiment the animals were killed by i.v. overdose of anaesthetic (Sagatal, 600 μl/animal).

Plasma concentrations of ACTH, 17β-estradiol and progesterone were measured using highly sensitive specific commercially available assay kits (Euro-diagnostics, Netherlands and Serozyme, USA, respectively). Plasma corticosterone

was measured by a specific scintillation proximity assay. In addition we measured corticosterone binding globulin (CBG) concentrations, the plasma protein that binds approximately 90% of corticosterone in the circulation, since a number of studies have indicated that estrogen increases its level (Gala & Westphal, 1965; Seal & Doe, 1965). A binding assay was used to measure the CBG levels. For details see Chapter 2.

7.3 Results

7.3.1 Influence of estradiol and progesterone on the HPA axis secretory response to FS stress.

The basal plasma ACTH concentrations did not differ between the ovarian steroid- and the vehicle-treated groups (Fig. 7.1a). At 5 min after the FS stress the plasma ACTH levels of both groups were significantly increased 4- and 5-fold, steroid and vehicle-treated group, respectively, ($p < 0.001$). The maximum response occurred at 5 min post-stress and was not significantly different between the two groups, and at 60 min post-stress plasma ACTH levels had returned to basal.

The basal corticosterone concentrations did not differ between the steroid- and vehicle-treated animals (Fig 7.1b). FS stress significantly increased the plasma levels in both groups ($p < 0.001$). The maximum response occurred at 15 min post-stress in the vehicle-treated group while in the steroid group the maximum corticosterone levels were not reached until 30 min after the end of the FS stress, however, this was not significantly different from the 5 or 15 min post-stress level. At 60 min post-stress the plasma corticosterone concentration in both groups was still elevated, however, they were not significantly different from basal values.

7.3.1.1 Influence of ovarian steroids on plasma CBG levels

The circulating CBG concentrations did not significantly differ between the steroid and vehicle-treated groups (14.2 ± 1.3 and 13.6 ± 1.8 pmol [^{3}H]corticosterone bound/mg of protein , respectively).

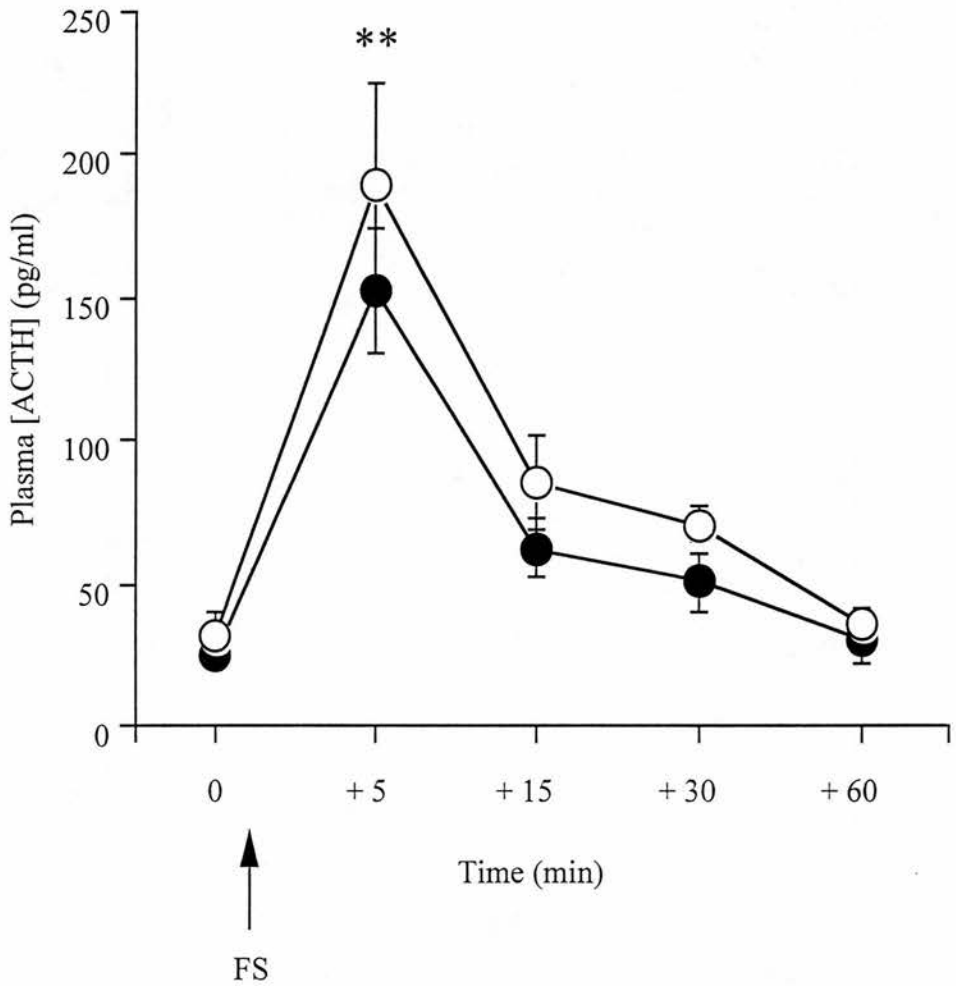


Fig 7.1a: Effect of combined chronic estradiol and progesterone treatment with silastic capsules implanted subcutaneously for 16 and 14 days, respectively, on the ACTH secretory response to forced swim (FS) stress of ovary-intact female rats. Vehicle- (○) & ovarian steroid-treated (●), n = 8 and 10, respectively. Two basal samples were collected 30 min apart; however, these were not significantly different so were pooled and expressed as a single basal sample (time 0). Then 30 min later each rat was exposed to FS stress for 90 s and blood samples were collected 5, 15, 30 & 60 min post-stress. ANOVA followed by Newman-Keul test: ** $p < 0.001$ vs. pre-swim levels in both groups.

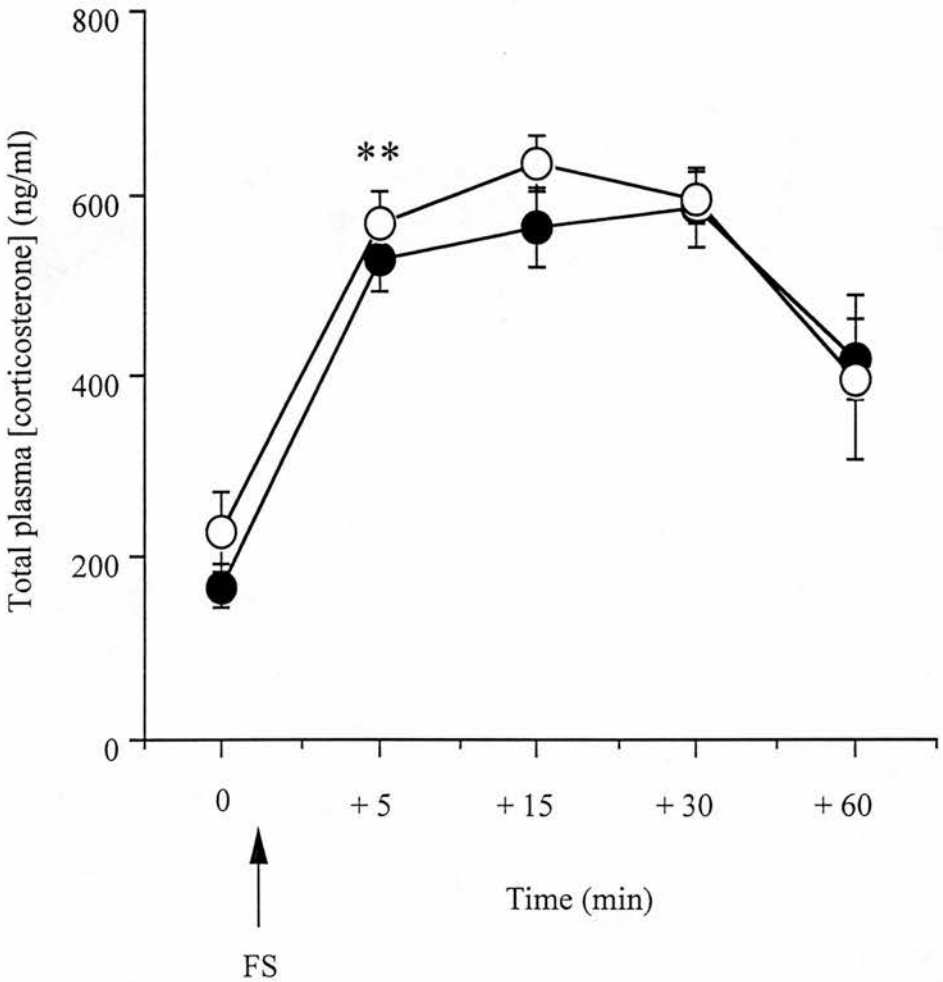


Fig 7.1b: Effect of combined chronic estradiol and progesterone treatment with silastic capsules implanted subcutaneously for 16 and 14 days, respectively, on the corticosterone secretory response to forced swim (FS) stress of ovary-intact female rats. Vehicle- (○) & ovarian steroid-treated (●), n = 8 and 10, respectively. Two basal samples were collected 30 min apart, however, these were not significantly different so were pooled and expressed as a single basal sample (time 0). Then 30 min later each rat was exposed to FS stress for 90 s and blood samples were collected 5, 15, 30 & 60 min post-stress. ANOVA followed by Newman-Keul test: ** $p < 0.001$ vs. pre-swim levels (time 0) in both groups.

7.3.1.2 Plasma estradiol and progesterone concentrations

The plasma estradiol concentrations approximately 16 days after the implantation of the capsule and at the end of the experiment were 169.7 ± 25.6 and 132.0 ± 47.1 pg/ml, respectively (Fig 7.2a).

The plasma progesterone concentrations 14 days after the implantation of the capsules and at the end of the experiment were 87.8 ± 15.6 and 60.0 ± 8.5 ng/ml, respectively (Fig 7.2b).

The circulating levels of estradiol and progesterone in the vehicle-treated group were 63.9 ± 7.1 pg/ml and 4.5 ± 1.2 ng/ml, respectively (pooled for 14/16 day and final day of experiment; see fig 7.2a & 7.2b).

7.3.2 The influence of progesterone withdrawal on the HPA axis response to FS stress.

The basal plasma ACTH concentrations did not differ among the ovarian steroid-treated, progesterone-withdrawal and vehicle groups (Fig 7.3a). Following exposure to FS stress the plasma ACTH concentration in all groups increased significantly ($p < 0.001$: 8- and 7- and 10-fold for steroid-treated, progesterone-withdrawal and vehicle group, respectively) at 5 min post-stress with the response not being significantly different amongst the three groups.

The basal plasma corticosterone concentrations did not differ among the steroid, progesterone-withdrawal and vehicle groups (215.4 ± 88.5, 196.7 ± 59.8 and 303.1 ± 100.1 ng/ml, respectively) (Fig 7.3b). The FS stress significantly increased the plasma concentrations ($p < 0.001$: increased after 5 min) and the response were not significantly different amongst the three groups.

7.4 Discussion

Although the sexual dimorphism in the activity of the HPA axis has been known for a number of years and it is generally accepted that estrogens are important in this phenomenon we did not find a significant difference in the basal plasma ACTH and corticosterone levels between the ovarian steroid-treated and the vehicle

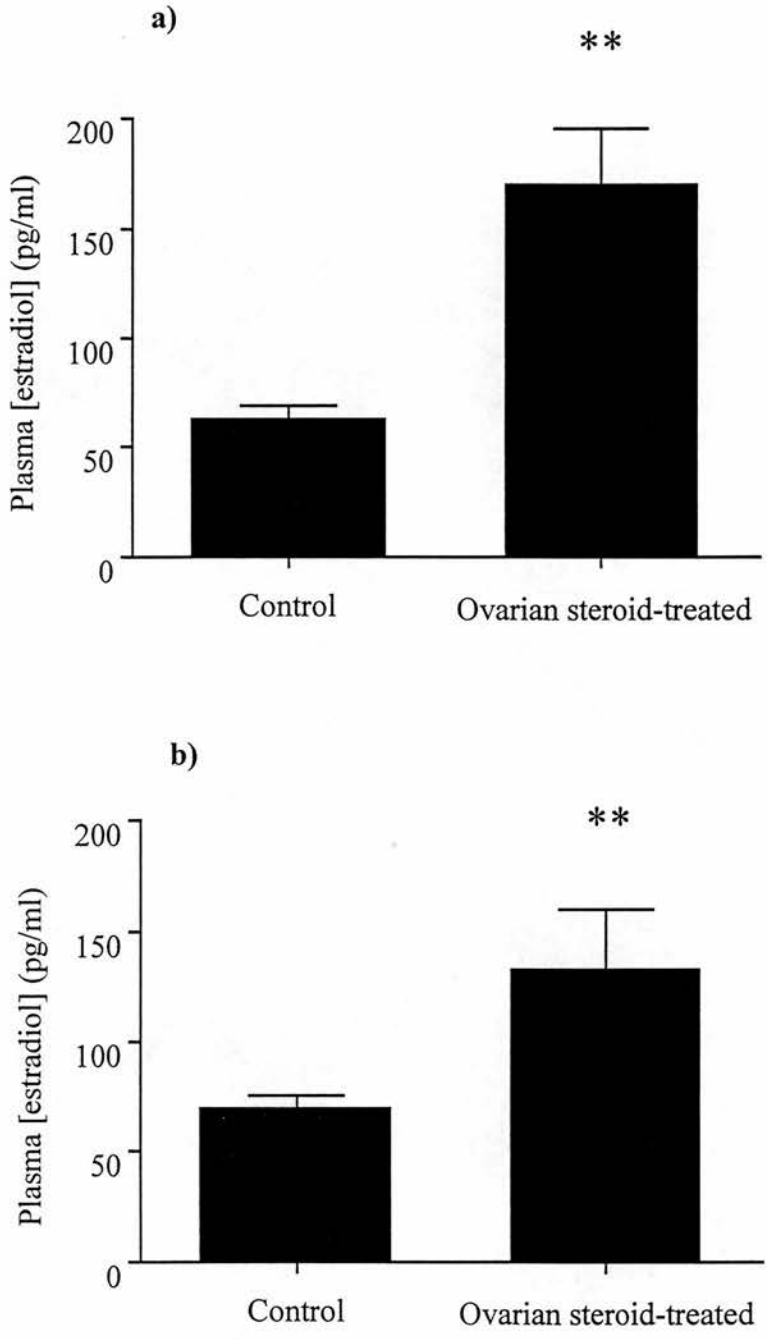


Fig 7.2a: Plasma estradiol concentrations **a)** two weeks after s.c implantation of estradiol-containing, progesterone-containing or vehicle silastic capsules. **b)** at the end of the experiment (21 days after capsule implantation). Students t test: ** $p < 0.001$ vs. vehicle control. Data expressed as group means ± S.E.M., n = 8 in each group.

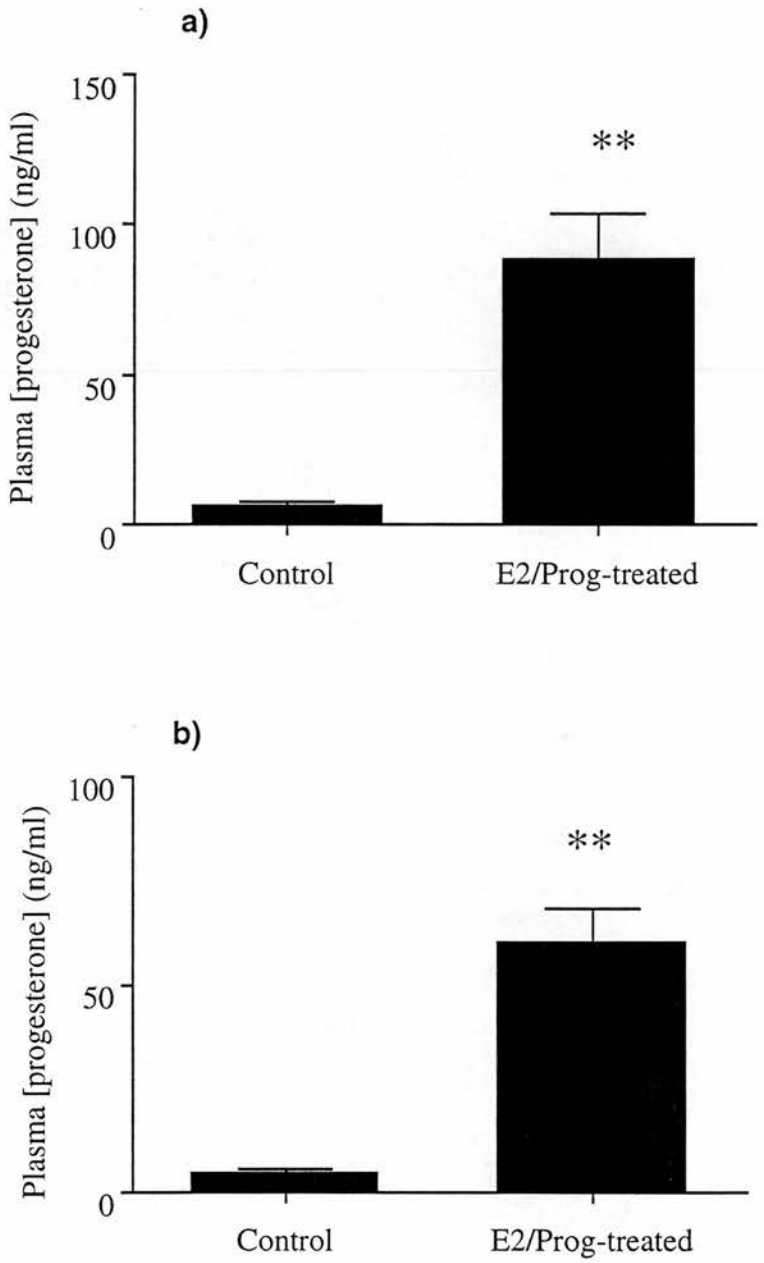


Fig 7.2b: Plasma progesterone concentrations **a)** two weeks after s.c implantation of estradiol-containing, progesterone-containing or vehicle silastic capsules. **b)** at the end of the experiment (21 days after capsule implantation). Students t test: ** $p < 0.001$ vs. vehicle control. Data expressed as group means ± S.E.M., n = 8 in each group.

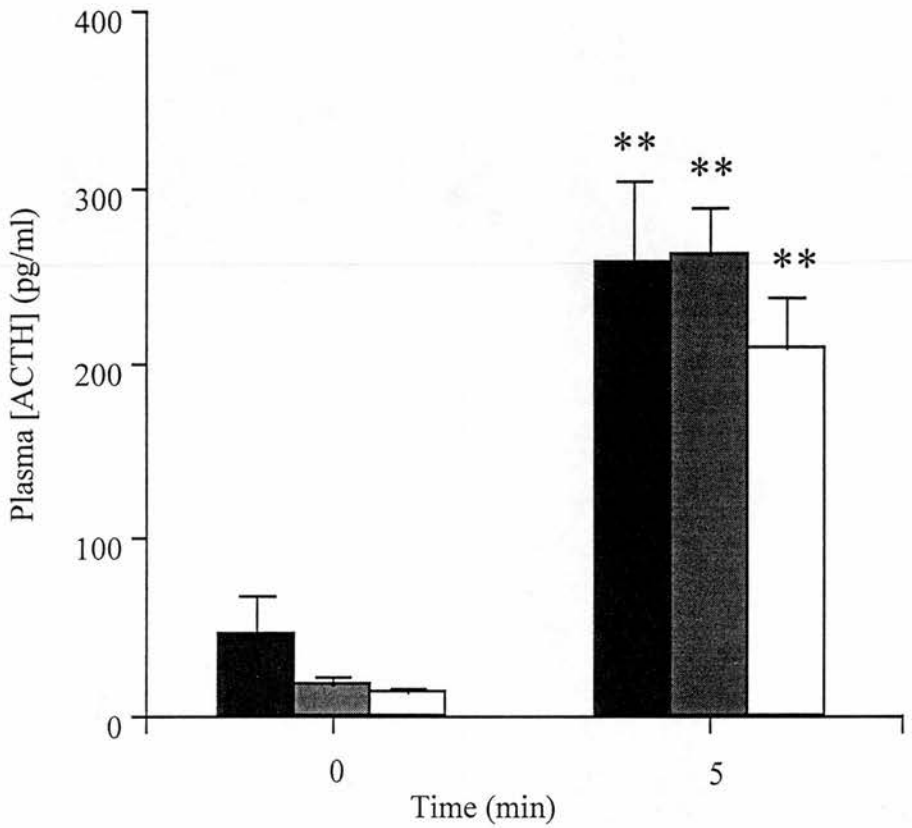


Fig 7.3a: Effect of chronic estradiol and progesterone treatment with the removal of the progesterone capsules two days prior to the experiment on the ACTH secretory response to forced swim (FS) stress of ovary-intact female rats. Vehicle-(■), ovarian steroid-treated (▩) & ovarian steroid treated with progesterone withdrawal (□) n = 5, 6 & 7, respectively. Two basal samples were collected 30 min apart; however, these were not significantly different so were pooled and expressed as a single basal sample (time 0). Then 30 min later each rat was exposed to FS stress for 90 s and blood samples were collected 5 min post-stress. ANOVA followed by Newman-Keul test: ** $p < 0.001$ vs. pre-swim levels in all groups.

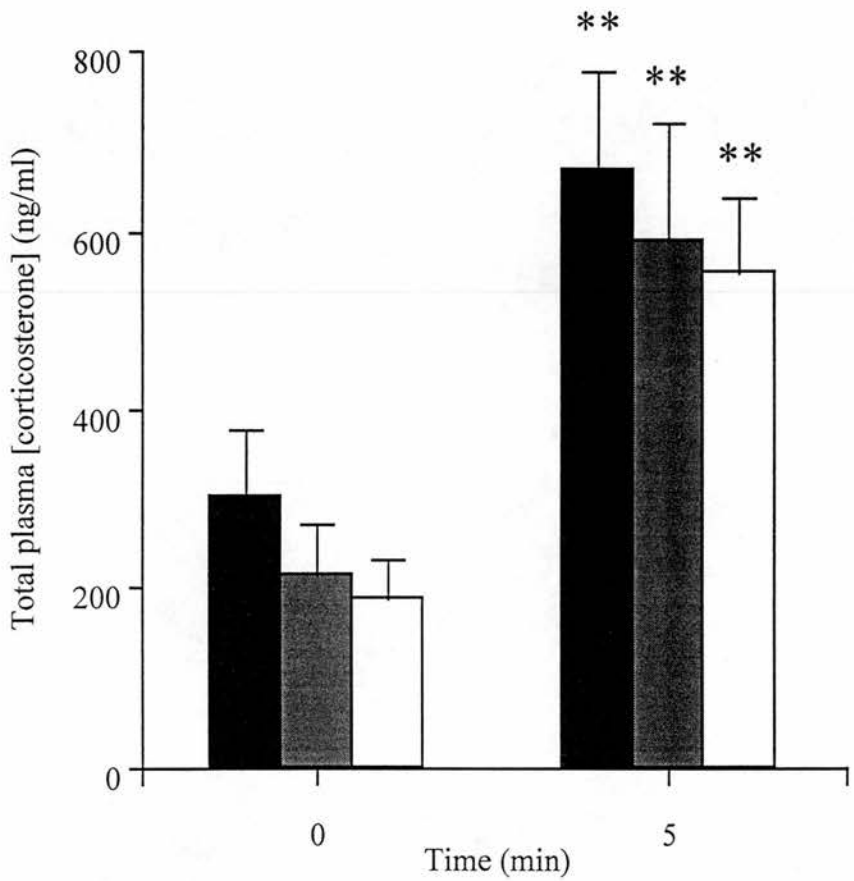


Fig 7.3b: Effect of chronic estradiol and progesterone treatment with the removal of the progesterone capsules two days prior to the experiment on the corticosterone secretory response to forced swim (FS) stress of ovary-intact female rats. Vehicle- (■), ovarian steroid-treated (▩) & ovarian steroid treated with progesterone withdrawal (□) n = 5, 6 & 7, respectively. Two basal samples were collected 30 min apart; however, these were not significantly different so were pooled and expressed as a single basal sample (time 0). Then 30 min later each rat was exposed to FS stress for 90 s and blood samples were collected 5 min post-stress. ANOVA followed by Newman-Keul test: ** $p < 0.001$ vs. pre-swim levels in all groups.

groups. This is in agreement with the report of Viau & Meaney, (1991) who found that in intact female rats neither basal ACTH or corticosterone concentrations varied across the estrous cycle. However, other studies have found that although the morning ACTH and corticosterone levels did not differ, in samples taken in the late afternoon or early evening of proestrus there were elevated levels of both (Carey *et al.*, 1995). This was confirmed by measurements of the free plasma corticosterone fraction (Carey *et al.*, 1995). Thus the reason why we did not see an elevation in our basal ACTH and corticosterone measurements may partly be due to the fact that all our basal samples were collected in the morning.

In response to 90 sec of FS stress the plasma levels of both ACTH and corticosterone increased significantly in all groups. The maximum ACTH response occurred 5 min post-stress and the magnitude was similar in both groups. The maximum plasma corticosterone concentrations in the vehicle-treated group were achieved 15 min post-stress, which is consistently seen in all our stress paradigm studies, while the steroid-treated group's corticosterone levels did not reach a maximum until 30 min post-stress. It may be postulated that this difference may reflect a reduction in the central forward drive to the axis since a previous report found that chronic estrogen treatment decreased hypothalamic CRF content (Haas & George, 1989). Alternatively, it may reflect a prolonged response due to an attenuation of the delayed negative feedback. It has previously been demonstrated that in estradiol-replaced OVX rats there is a decrease in GR binding and transcription (Burgess & Handa, 1992; Pfieffer & Barden, 1987) and that both estradiol and progesterone treatment in OVX rats have effects on MR (Burgess & Handa, 1993; Carey *et al.*, 1995). In general, MR with its high affinity for corticosterone is proposed to be solely responsible for regulating morning ACTH levels (Dallman *et al*, 1989a) while GR is important in controlling the peak basal and stress ACTH levels (Bradbury *et al.*, 1991), however, evidence exists which suggests that occupancy of MR is necessary in the regulation of both peak basal and stress levels of ACTH (Bradbury *et al.*, 1994; Ratka *et al.*, 1989). If either of these were the cause a delay would be expected for plasma ACTH levels to reach a maximum, but this was not found. More importantly, however; is that plasma corticosterone

concentrations at both the 15 min and 30 min time points were not significantly different from the 5 min post-stress value.

By 60 min the plasma ACTH levels had returned to basal values, while the corticosterone levels in both groups remained elevated. Similarly, a previous report found that the clearance rates of ACTH and corticosterone were not different in OVX steroid-replaced rats (Viau & Meaney, 1991). In support of this we found no difference in the circulating levels of CBG between groups. Again our data is in disagreement with previous studies. Several groups have reported that estrogen treatment in both humans and rats increase plasma levels of CBG (Blomback *et al.*, 1983; Gala & Westphal, 1965)

The lack of influence of the ovarian steroids on both the basal activity and stress activation of the HPA axis question whether the circulating levels of estradiol and progesterone in the virgin animals with implants were comparable to those found in pregnancy. However, we can discount this as initial observations during jugular cannulation surgery strongly indicated that the plasma levels of estradiol and progesterone were elevated since mammary tissue development was marked and, measurement of plasma ovarian hormone concentrations showed that circulating levels were similar to those previously reported during pregnancy (Shiakh, 1971; Wiest, 1970).

During the last few days of pregnancy the circulating levels of progesterone dramatically fall while estradiol levels remain consistently high until parturition. This fall in plasma progesterone is due to the degeneration of the corpus luteum, the major source of progesterone during pregnancy in rodents (Taya & Greenwald, 1981). A recent report by Windle et al, (1997a) suggested that the hyporesponsiveness of the HPA axis to stress of lactating rats may be due to this fall in plasma progesterone. In our study, we did not detect a difference in the maximum secretory responses to acute stress in the progesterone withdrawal animals. However, the measurements were not extended beyond the 5 min post-stress. However, we have found that reduced HPA axis responses to a stressor are evident from day 16 of pregnancy, some 5 days before collaspe of the corpus lutea (Neumann *et al*, 1998). It is important to note that the majority of chronic ovarian steroid studies used OVX rats

replaced with estradiol and/or progesterone and OVX rats as controls. In our study ovary-intact rats were used to enable comparison with the studies on pregnant rats in which intact virgins were used as controls. Thus, interpretation of previous studies on OVX rats is difficult in the context of changes in pregnancy.

In conclusion, from our study it seems that estradiol and/or progesterone, at plasma concentrations equivalent to those found in pregnancy, are not mediators of the hyporesponsiveness of the HPA axis seen during late pregnancy.

GENERAL DISCUSSION

My PhD project examined the responsiveness of the HPA axis in late pregnancy and investigated possible mechanisms which may underlie the demonstrated hyporesponsivess of the HPA axis to acute stress in the late pregnant rat.

8.1 Attenuated HPA axis stress response during pregnancy

In this study we demonstrated that in late pregnancy the HPA axis of the rat is less responsive to acute stress (Fig 3.1a & Fig 3.1b) which is in agreement with the observation of Neumann *et al* (1998). In addition, we propose that this phenomenon is not dependent on either the stress paradigm or the rat strain used indicating that this may be an important physiological adaptation in pregnancy. This attenuated HPA axis stress responsiveness is similar to the suppression previously seen in lactating rats (Stern *et al.*, 1973; Walker *et al.*, 1992; Windle *et al.*, 1997). A recent report examining the central mRNA expression of the immediate early gene, *c-fos* in response to acute stress (da Costa *et al.*, 1996) found that in both late pregnant and lactating rats the expression was significantly lower, not only in the parvocellular PVN, but also in the medial amygdala, a subnucleus of the amygdaloid complex, and the lateral septum following stress. Interestingly, both these structures possess indirect connections to the PVN (Sawchenko & Swanson, 1983) and are known to influence the activity of the HPA axis (Dunn & Whitener, 1986). Since the expression of c-*fos* mRNA is considered a marker of neuronal activation this result suggests a common mechanism of reduced activation of specific afferent pathways to the PVN may be involved in producing the hyporesponsiveness of the HPA axis in both the pregnnant and lactating rat. However, the HPA axis is a multilevel system and is regulated at these various levels by numerous factors, therefore other regulatory mechanisms downstream from the afferent pathways cannot be ruled out in contributing to the suppressed stress-induced HPA axis activity during pregnancy.

The reduced responsiveness of the HPA axis to stress might possibly be an additional adaptive mechanism to protect the fetus from excess exposure to

glucocorticoids. In addition it may be involved in maintaining maternal energy stores required for the support of the developing fetus and in preparation for parturition.

Thus the late pregnant rat like the lactating rat has an attenuated HPA axis response to acute stress which involves modulation of afferent pathways to the parvocellular neurones of the PVN; however, additional modulation may occur at the hypothalamic and anterior pituitary level.

8.2 Changes at the level of the PVN and in the responsiveness of the anterior pituitary corticotroph during pregnancy

We confirmed the finding of Douglas & Russell (1994) that on day 21 of pregnancy basal CRF mRNA expression in the parvocellular neurones of the PVN was significantly reduced; although not earlier in pregnancy (Fig 4.2). This is interesting since the suppressed stress response is seen from day 15 of pregnancy (Neumann *et al.*, 1998), suggesting that this reduced CRF mRNA expression is not the major contributor to this attenuation. We also examined the expression of parvocellular AVP mRNA since under certain circumstances, such as following adx, AVP becomes the predominant ACTH secretagogue (Albeck *et al.*, 1994; Holmes *et al.*, 1986) indicating that not only does CRF drive ACTH release from the anterior pituitary but has a permissive action on AVP (Plotsky *et al.*, 1985). However, we did not find a significant change in AVP mRNA levels although it tended to decrease (Fig 4.2). Thus, in late pregnancy there appears to be either an increased inhibitory or decreased excitatory influence on the parvocellular neurones of the PVN which may be mediated directly or indirectly.

The high levels of circulating glucocorticoids that occur during pregnancy (Atkinson & Waddell, 1995; Nolten & Kueckert, 1981) may be responsible for an increased inhibitory influence on the PVN. Glucocorticoids are well known to inhibit the parvocellular CRF neurones directly (Kovacs & Makara, 1988; Kovacs & Mezey, 1987; Kovacs *et al.*, 1986). However, our AVP mRNA data do not support a direct effect of corticosterone at the level of the PVN. Parvocellular AVP-containing neurones are highly sensitive to feedback by glucocorticoids and removal of endogenous corticosterone causes a greater upregulation in AVP expression compared to CRF (Albeck *et al.*, 1994). Thus, if the high levels of corticosterone

were directly inhibiting gene expression at the level of the PVN we would have expected to observe a comparable decrease in both CRF and AVP mRNA expression. Glucocorticoids also inhibit parvocellular CRF neurones indirectly via actions at extrahypothalamic sites. The best characterised being the hippocampus (Herman *et al.*, 1989, 1992b; Salpolsky *et al*, 1984, 1989).The hippocampus has been shown to modulate basal HPA axis activity as well as the stress response (Bradbury & Dallman, 1989). Thus, corticosterone may be influencing parvocellular gene expression by acting indirectly via extrahypothalamic sites such as the hippocampus.

Alternatively, a decreased excitatory input from the catecholaminergic cell bodies in the brainstem to the parvocellular PVN (Cunningham & Sawchenko, 1988) may account for the decreased CRF expression. These cell groups appear to have a tonic stimulatory input to the PVN CRF neurones since transection of this afferent pathway results in a decrease in parvocellular CRF mRNA levels (Kiss *et al.*, 1996). Interestingly, these catecholaminergic cell groups, including the NTS which has a major projection to the parvocellular CRF neurones, all express GR (Fuxe *et al.*, 1985; Reul & de Kloet, 1986). Thus, glucocorticoids may modulate PVN CRF gene expression indirectly at the level of the brainstem by influencing the activity of these catecholaminergic cell groups.

In both human and rat pregnancy the sensitivity of the anterior pituitary *in vivo* to exogenous CRF is diminished (Goland *et al.*, 1990; Neumann *et al.*, 1998). This insensitivity may be partly explained by our observation of reduced density of CRF binding sites in the anterior pituitary with progressing pregnancy (Fig 4.5a). However, in order to ascertain whether this decreased binding reflects a decrease in receptor affinity (kd) or a decrease in receptor number (B_{max}) subsequent receptor binding studies using Scatchard analysis will have to be carried out. CRF has been shown to possess the ability to downregulate its own receptors (Reisine & Hoffman, 1983), however, increased release of CRF into the HPB is not supported by our parvocellular PVN CRF *in situ* hybridisation data. Alternatively, glucocorticoids have been shown *in vitro* to decrease CRF binding to anterior pituitary membranes (Hauger *et al.*, 1987) and *in vivo* to reduce the mRNA expression of $CRFR_1$ (Makino *et al.*, 1995a). Although the precise mechanism of this reduction is unknown it is proposed to be an additional inhibitory mechanism through which glucocorticoids

regulate the release of ACTH (Hauger *et al.*, 1987). An interesting finding was the decrease in CRF binding in the intermediate lobe of the pituitary gland on day 21 of pregnancy although the relevance of this reduction on the responsiveness of the anterior pituitary is unknown. It had previously been shown that these CRF receptors are not affected by either the removal of endogenous glucocorticoids or by replacement with dexamethasone (De Souza *et al.*, 1985b). However, the existence of an independent source of CRF in the intermediate lobe has been proposed (Saavedra *et al.*, 1984) which is proposed to regulate peptide secretion from the intermediate lobe. Therefore increases in this CRF pool may result in receptor downregulation; however, it is not known whether this pool increases during pregnancy.

The *in vitro* cAMP accumulation data showing a reduced cAMP response to exogenous CRF during late pregnancy (Fig 4.4c & Fig 4.5a) complements the CRF binding data (Fig 4.3b). The discrepancy between the levels of cAMP accumulated and the density of CRF binding in the anterior pituitary prior to day 20 of pregnancy: a 50% decrease in CRF binding, without producing a concomitant decrease in cAMP accumulation, probably reflects the large CRFR pool which exists in the anterior pituitary (King & Baertschi, 1990). The demonstration of a reduced cAMP accumulation in acutely dispersed anterior pituitary cells clearly demonstrated that the decreased responsiveness of the anterior pituitary corticotroph to CRF does not require cell to cell contact. One possible mechanism, may involve lipocortin I, the Ca^{2+} and phospholipid binding protein, which is expressed in the anterior pituitary and is induced by steroid-treatment (Smith *et al.*, 1990a). It has been proposed to mediate some of the inhibitory effects of corticosteroids (Flower, 1988).

Thus, decreased CRF mRNA expression in the PVN and in the density of CRFR binding sites in the anterior pituitary leading to decreased cAMP accumulation are likely to contribute to the stress hyporesponsiveness of the HPA axis during pregnancy.

Future experiments

As mentioned in Chapter 4 it would be useful to know whether CRF and/or AVP gene expression in response to an acute stress is reduced in the late pregnant

animals. The best way to determine this would be to carry out an *in situ* hybridisation study using an intronic probe, which allows detection of the primary transcripts and would provides a direct measurement of transcription.

As cAMP is an ubiquitous second messenger it is inappropriate to directly extrapolate it to the amount of ACTH being produced by these cells. Therefore, to support and strength the *in vitro* cAMP data it is important to measure the amount of ACTH being secreted by the anterior pituitary corticotrophs following exposure to exogenous CRF.

8.3 The glucocorticoid negative feedback signal during pregnancy

The sensitivity of the HPA axis to the glucocorticoid feedback signal during pregnancy is species-dependent. Owens *et al* (1987) reported a reduced feedback sensitivity in humans that extends up to three weeks postpartum while Keller-Wood (1996) found it unchanged in the sheep, but no such data exist in the rat. Thus, the attenuated stress response may potentially reflect an enhanced glucocorticoid feedback signal on the HPA axis of the pregnant rat. However, neither MR or GR mRNA levels varied in the hippocampus or in the parvocellular PVN, respectively, during pregnancy compared to virgin rats (Fig 5.1a, 5.1b & 5.1c). Although a small increase in GR expression was detected in the dentate gyrus of the hippocampus which may indirectly influence the PVN via a GABAergic input (Cullinan *et al.*, 1993) the physiological impact of this is unknown. Interestingly other studies examining GR expression have found that changes in mRNA levels do not necessarily reflect changes in receptor binding or function (Bohn *et al.*, 1994; Chao *et al.*, 1989). Therefore it would be pertinent to undertake further studies using either immunohistochemical or receptor binding techniques to clarify the situation.

The enzyme 11β-HSD1 regulates the access of glucocorticoids to GR in numerous tissues (Moisan *et al.*, 1990b; Whorwood *et al.*, 1992) and is located in several tissues involved in the regulation of the HPA axis including the anterior pituitary, PVN and hippocampus (Moisan *et al.*, 1990a; Seckl *et al.*, 1993) and thus may be involved in regulation of the glucocorticoid feedback signal (Seckl *et al.*, 1993). In the rat , *in vitro* enzyme activity increased during pregnancy in the PVN and to a lesser extent in the anterior pituitary (Fig 5.2a). Accumulating evidence

using clonal cell lines and primary cell culture indicate that this isoform acts predominantly as a reductase *in vivo.* Thus, we postulated that the enhanced dehydrogenase activity *in vitro* may *in vivo* act as a reductase resulting in an increased local production of corticosterone, particularly in the PVN which may be responsible for the decreased parvocellular CRF mRNA expression. To test the potential physiological influence of central 11β-HSD on the stress reactivity of the HPA axis we inhibited the enzyme by giving an i.c.v. infusion of glycyrrhetinic acid (GA), the active component of liquorice which is a potent inhibitor of the enzyme (Stewart *et al.*, 1987; Edwards *et al.*, 1988). We were unable to demonstrate a significant effect fof GA on the ACTH secretory response to acute stress (Fig 5.3a).

To test the full responsiveness of the HPA axis to glucocorticoid feedback we pharmacologically adrenalectomised with metyrapone and aminoglutethimide and examined the sensitivity of the mechanisms regulating ACTH secretion to exogenous corticosterone (Fig 5.4a). The HPA axis of the pregnant rat responded in a similar manner to removal of endogenous glucocorticoids as the virgin rat. Thus indicating that the HPA axis of the pregnant rat is not under a greater inhibitory influence of glucocorticoids. Interestingly, for 30 min after administration of exogenous corticosterone the plasma ACTH levels in the pregnant rats, unlike in the virgin controls, actually showed reduced feedback sensitivity. This result indicates that pregnant rats may be more resistant to the fast feedback effects of glucocorticoids which occur as the plasma level of the steroid is rising. Similarly, a recent report in pregnant sheep found that basal plasma ACTH levels were less sensitive to inhibition by exogenous corticosterone at 20 min after administration (Keller-Wood, 1996). The physiological relevance of this reduced sensitivity is unclear since fast feedback has been shown to increase the rate of decline of plasma ACTH levels following stress rather than decrease the peak levels achieved (De Souza & Van Loon, 1989). However, it is clear that enhanced negative feedback is not involved in the suppression of the acute stress response in pregnancy and that pregnant rats appear to have an unchanged sensitivity to intermediate glucocorticoid feedback.

Future experiments

Although the *in situ* hybridisation studies for MR and GR did not show a difference in gene expression between pregnant and virgin animals in the hippocampus it has previously demonstrated that mRNA levels do not always reflect what occurs at the protein level. Therefore, to conclusively show that there is no difference it might be desirable to use receptor binding assays.

In order to directly show that inhibition of central 11β-HSD infuences the activity of the HPA axis duringa stress administration of GA directly into the PVN may provide a clear-cut answer.

8.4 The influence of central endogenous opioid systems on the HPA axis during pregnancy

Endogenous opioid peptides (EOPs) such as β-endorphin, met- and leu-enkephalin and dynorphin have a wide central distribution (for review see Khachaturian *et al.*, 1985). Since during pregnancy the levels of central EOPs change, including an increase in the hypothalamic content of β-endorphin (Wardlaw & Frantz, 1983) and in late pregnancy EOPs restrain stimulation of oxytocin neuronal activity and secretion by brainstem inputs (Douglas *et al.*, 1995) there may be a common, central opioid mechanism inhibiting neuroendocrine responses in pregnancy. Similarly central opioid peptides may have a role in causing the suppression of the HPA axis stress response in pregnancy. In our study administration of the opioid antagonist naloxone (nlx) did not influence the basal plasma levels of ACTH or corticosterone in either day 21 pregnant or virgin rats (Fig 6.1a,b). This indicates there is no endogenous opioid tone on the basal activity of the HPA axis. In contrast, several other studies have shown that nlx stimulates CRF and ACTH secretion *in vivo* (Eisenberg, 1980; Jezova *et al.*,1982; Nikolarakis *et al.*, 1987; Plotsky, 1986). This discrepancy may reflect differences in the route of administration of nlx (Iyengar *et al.*, 1986; Plotsky *et al.*, 1986) or differences in sampling times. It is worth noting that the other studies used male animals. Although no data exists to suggest a sexual dimorphism in the influence of EOP it has been previously reported that a sexual dimorphism exists in the sensitivity to endogenous opioids of oxytocin secretion in response to a stress (Carter *et al.*, 1986; Carter &

Lightman, 1987). Thus, a similar phenomenon may occur with regard to basal HPA axis activity. In virgin rats EOPs are involved in the stimulation of the HPA axis following an acute stress since nlx treatment significantly decreased the plasma ACTH response to forced swimming (Fig 6.1a). The site(s) of action are unknown although it is more likely to be a central mechanism since the expression of opioid peptide receptors is absent or extremely low in the anterior pituitary (Boersma *et al.*, 1994; Calogero *et al.*, 1996). Thus, central EOPs may enhance stress-induced ACTH secretion by acting at the level of the parvocellular CRF neurones and/or at extrahypothalamic sites, including the catecholaminergic brainstem cell groups since they have a large central distribution. In contrast, during late pregnancy this stimulatory effect of EOP on the stress response is lost and this may reflect a desensitisation of the system possibly at the level of the hypothalamus since their levels rise with the progress of pregnancy (Wardlaw & Frantz, 1983). Alternatively, the small increase in the maximum ACTH response to stress in the pregnant animals following nlx treatment may actually suggest that during pregnancy there may be a switch from a stimulatory opioid to an inhibitory influence on the stress responsiveness of the HPA axis. Thus, central EOPs may be involved in the suppression of the HPA axis to acute stress in pregnancy.

Future experiments

In the above study at the dosage used nlx acted as a non-specific opioid antagonist so we were unable to determine the opioid peptide(s) involved. possible. *In vivo* studies using specific opioid antagonists would allow the determination of the opioid peptide(s) involved in influencing the HPA axis, particularly in the virgin rats.

8.5 The influence of ovarian hormones on the HPA axis during pregnancy

A close relationship exists between the HPA axis and the hypothalamic-pituitary-ovarian (HPO) axis. In contrast to the primarily inhibitory influence of the HPA axis on reproductive function, the vast majority of studies have demonstrated that estradiol treatment in ovariectomised rats has a stimulatory role in HPA axis regulation (Buckingham *et al.*, 1978; Carey *et al.*, 1995; Raps *et al.*, 1971; Viau & Meaney, 1991). Ovarian hormones, and estradiol in particular, are thought to be

responsible for the sexual dimorphism of the activity of the HPA axis, with females exhibiting a higher activity under basal conditions and in response to stress (Critchlow *et al.*, 1963; Kitay, 1961). However, some studies suggest that chronic treatment with estradiol may cause an inhibitory influence by decreasing the ACTH releasing activity of median eminence extract and hypothalamic CRF content (Haas & George, 1989; Kitay, 1963). The influence of progesterone is not so well-defined; at low concentrations it acts as an antiglucocorticoid while at higher concentrations it has partial agonistic activities (Keller-Wood *et al.*, 1988). The circulating estradiol and progesterone in the treated animals reached levels comparable to those previously reported in pregnancy (Shiakh, 1971; Weist, 1970). There was no difference in the basal plasma levels of either ACTH and corticosterone between the groups (Fig 7.1a & Fig 7.1b). This was not unexpected as our blood sampling was carried out in the morning and previous reports suggest that the enhancing effects of ovarian steroids are seen only on the elevated levels in the late afternoon or early evening (Buckingham *et al.*, 1978; Carey *et al.*, 1995). The responses of the ovarian hormone- and the vehicle-treated animals to the forced swimming stress paralleled each other, reaching similar maximum plasma levels of ACTH and corticosterone.

Several of our observations have occurred on day 21 of pregnancy, the day prior to parturition, including the decreased CRF mRNA expression in the PVN and the decreased CRF receptor density in the anterior pituitary. At this time the plasma levels of progesterone have dramatically fallen due to the degeneration of the corpora luta, the major source of progesterone in rat pregnancy. When we looked at the effect of withdrawal of progesterone on the responsiveness of the HPA axis to stress we found no difference. Thus, ovarian hormones do not appear to be involved in inducing this decreased HPA axis responsiveness in pregnancy.

Biological implications for the mother of abnormal functioning of the HPA axis

Both physiological conditions such as aging (Hatzinger *et al.*, 1996) and pathophysiological conditions such as depression (Holsboer & Barden, 1996) involve perturbations of the HPA axis. Similarly, human pregnancy is associated with an increased incidence of mental illness and mood disturbance in both the immediate antenatal and postnatal periods. The changes in circulating levels of many hormones

during pregnancy and their dramatic fall at parturition has led investigators to examine their relationship with mood disturbance. Much interest has focussed on the potential changes in the HPA axis during pregnancy and the development of postnatal depression. Similar disturbances of HPA axis activity to those seen in endogenous depression appear to occur in pregnancy. In both situations basal concentrations of corticosteroids are elevated, while a diurnal rhythm is maintained (Atkinson & Waddell, 1995; 1988; Nolten *et al.*, 1980); in humans, the suppression of plasma cortisol by dexamethasone is blunted; and the responsiveness of the anterior pituitary to injected CRF is depressed in both humans and rats (Gold *et al.*, 1988; Neumann *et al.*, 1998). However, whether the mechanism(s) of these changes is/are identical is not clear. Gold *et al* (1988) postulated that the endocrinological abnormalties observed in endogenous depression were due to hypersecretion of CRF from the hypothalamus. This notion is supported by the finding that normal subjects given a continuous infusion of CRF exhibit a similar pattern and magnitude of hypercortisolaemia to that seen in major depression (Schulte *et al.*, 1985). However, our findings of decreased CRF gene expression in the PVN during pregnancy in the rat suggests that enhanced hypothalamic CRF secretion does not occur during pregnancy. In humans, secretion of CRF from the placenta into the circulation has been postulated to influence the maternal HPA axis (Waddell, 1993) however, measurements of the plasma levels of CRF during pregnancy do not correlate with the development of mood disorders (Smith *et al.*, 1990b). It is interesting to note that the abnormalities in HPA axis activity continue for several week after delivery when the plasma levels of CRF have returned to prepregnancy levels (Owens *et al.*, 1987) and altered HPA axis activity occurs in pregnancy in species such as rodents and sheep which do not possess elevated levels of plasma CRF during pregnancy (Linton *et al.*, 1986).

Several studies have examined the relationship between the disturbances in the HPA axis in pregnancy and the development of postnatal mood disturbances. The development of postnatal 'blues' was found to be significantly related to plasma cortisol levels measured at 38 weeks of pregnancy (Handley *et al.*, 1980). Another study looked at the relationship of abnormalities in the dexamethasone test preformed postnatally and the development of mood disorder; however, no

significant correlation was found (Smith *et al.*, 1990b). Newham *et al* (1984) examined the relationship between β-endorphin and postnatal mood and demonstrated a relationship between plasma levels of this opioid peptide at 38 weeks with the occurance of postnatal blues. Also Smith *et al* (1990b) found a significant correlation between between the fall in β-endorphin levels from 38 weeks to day 2 postpartum and the degree of mood disturbance in the first week. It should be noted that all these studies focussed on peripheral levels of cortisol and β-endorphin which may not represent the concentrations to which the neurones of the limbic system involved in regulating mood changes are exposed to.

The neuroendocrine response of the HPA axis is required for the survival of the organism during times of stress, therefore possessing a reduced stress response would be expected to be detrimental to the mother. However, we found that the pregnant rats survived acute experimental stress well and do not appear to be more anxious than nonpregnant rats in the elevated plus maze (Neumann *et al.*, 1998). One explanation would be that their high circulating levels of glucocorticoids are sufficient to maintain an adequate bodily response to stress. It would be of interest to examine how well pregnant rats cope with chronic stress.

I hope my work has contributed to the understanding of the mechanisms underlying the HPA axis stress hyporesponsiveness of the HPA axis to acute stress during late pregnancy.

However, my main wish is that I have generated as many questions as I have answered, thus providing the building blocks for further work in this area.

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Attenuated neuroendocrine responses to emotional and physical stressors in pregnant rats involve adenohypophysial changes

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1. The responsiveness of the rat hypothalamo–pituitary–adrenal (HPA) axis and hypothalamo–neurohypophysial system (HNS) to emotional (elevated plus-maze) and physical (forced swimming) stressors and to administration of synthetic corticotrophin-releasing hormone (CRH) was investigated during pregnancy and lactation. In addition to pregnancy-related adaptations at the adenohypophysial level, behavioural responses accompanying the neuroendocrine changes were studied.

2. Whereas basal (a.m.) plasma corticosterone, but not corticotrophin (adrenocorticotrophic hormone; ACTH), levels were increased on the last day (i.e. on day 22) of pregnancy, the stress-induced rise in both plasma hormone concentrations was increasingly attenuated with the progression of pregnancy beginning on day 15 and reaching a minimum on day 21 compared with virgin control rats. A similar attenuation of responses to both emotional and physical stressors was found in lactating rats.

3. Although the basal plasma oxytocin concentration was elevated in late pregnancy, the stress-induced rise in oxytocin secretion was slightly lower in day 21 pregnant rats. In contrast to vasopressin, oxytocin secretion was increased by forced swimming in virgin and early pregnant rats indicating a differential stress response of these neurohypophysial hormones.

4. The blunted HPA response to stressful stimuli is partly due to alterations at the level of corticotrophs in the adenohypophysis, as ACTH secretion in response to CRH *in vivo* (40 ng kg^{-1}, I.V.) was reduced with the progression of pregnancy and during lactation. *In vitro* measurement of cAMP levels in pituitary segments demonstrated reduced basal levels of cAMP and a lower increase after CRH stimulation (10 nM, 10 min) in day 21 pregnant compared with virgin rats, further indicating reduced corticotroph responsiveness to CRH in pregnancy.

5. The reduced pituitary response to CRH in late pregnancy is likely to be a consequence of a reduction in CRH receptor binding as revealed by receptor autoradiography. [^{125}I]CRH binding in the anterior pituitary was significantly reduced in day 11, 17 and 22 pregnant rats compared with virgin controls.

6. Anxiety-related behaviour of the animals as revealed by the time on and entries into the open arms of the elevated plus-maze was different between virgin and pregnant rats with decreased number of entries indicating increased anxiety with the progression of pregnancy (except on pregnancy day 18). The emotional behaviour, however, was not correlated with the neuroendocrine responses.

7. The results indicate that the reduced response of the HPA axis to stressors described previously during lactation is already manifested around day 15 of pregnancy in the rat and involves physiological adaptations at the adenohypophysial level. However, alterations in stressor perception at higher brain levels with the progression of pregnancy may also be involved.

In addition to its normal circadian variation, the hypothalamo–pituitary–adrenal (HPA) axis activity is altered by physiological or pathophysiological states that challenge the internal homeostasis of the organism. In addition to chronic stress (Fuchs & Flügge, 1994), psychiatric diseases (Holsboer & Barden, 1996) or ageing (Hatzinger, Reul, Landgraf, Holsboer & Neumann, 1996), pregnancy and lactation might represent a similar challenge due to the profound physiological adaptations occurring in the mother. Thus, in pregnancy there is increased sensitivity of the adrenal gland to corticotrophin (ACTH) and increased glucocorticoid secretion in several species (Dupouy, Coffigny & Magre, 1975; Carr, Parker, Madden, MacDonald & Porter, 1981; Waddell & Atkinson, 1994; Keller-Wood, 1996). However, there are no detailed studies regarding the responsiveness to stressors of the HPA axis reflected primarily by plasma ACTH and corticosterone concentrations, or of underlying mechanisms of possible pregnancy-related changes in HPA axis activity, or of associated behavioural alterations. The responsiveness of the HPA axis is reduced during lactation (see below), and we have now studied whether such altered responsiveness is established in pregnancy. Reduced reactivity of the maternal HPA axis may protect both the pregnant rat and her offspring from harmful excessive levels of glucocorticoids (for review, see Weinstock, 1997).

During lactation, the activity and regulation of the HPA axis are altered in ways that are dependent on the presence or suckling of the young (Walker, Lightman, Steele & Dallman, 1992; Windle *et al.* 1997). Thus, an increase in glucocorticoid secretion and a flattening of the diurnal rhythm in glucocorticoid secretion have been described (Stern, Goldman & Levine, 1973; Walker *et al.* 1992; Fischer, Patchev, Hellbach, Hassan & Almeida, 1995). In addition, secretion of ACTH from the anterior pituitary and, consequently, of cortisol (humans) and corticosterone (rats) from the adrenal glands is reduced during physical (Altemus, Deuster, Galliven, Carter & Gold, 1995; Walker, Trottier, Rochford & Lavallée, 1995), ether (Lightman & Young, 1989; Walker *et al.* 1992) and noise stress (Windle *et al.* 1997), and in response to cardiovascular stimuli (Keller-Wood, 1996). Furthermore, there are indications of altered emotional behaviour in lactating rats (Hard & Hansen, 1984; Walker *et al.* 1995).

Besides the well-described stress responses of the HPA axis there is also activation of the hypothalamo–neurohypophysial system (HNS), releasing either oxytocin or vasopressin or both from the neurohypophysis into blood, in response to various stressors (Lang, Heil, Ganten, Hermann, Unger & Rascher, 1983; Kasting, 1988; Wotjak, Kubota, Ganster, Liebsch, Neumann & Landgraf, 1996). During lactation, the stimulated oxytocin secretion is reduced in response to physical stress (Carter & Lightman, 1987; Lightman, 1992; Neumann, Pittman & Landgraf, 1995*b*; Walker *et al.* 1995) and hyperosmotic or pharmacological stimulation (Patel, Chowdrey & Lightman, 1991; Koehler, McLemore, Tang & Summy-Long, 1993; Neumann, Landgraf, Bauce & Pittman, 1995*a*). However, the reactivity of the oxytocinergic system during pregnancy has not been studied.

The present study was designed to test the secretory responses of the HPA axis and the HNS to the emotional and physical stressors of exposure to the elevated plus-maze and forced swimming, respectively, during pregnancy. We then studied the responsiveness to exogenous corticotrophin-releasing hormone (CRH) in the pregnant and virgin rats to seek changes at the level of the adenohypophysis in mechanisms regulating ACTH secretion from corticotrophs. We further sought modifications in CRH receptors or their coupling in corticotrophs by measuring cAMP production by anterior pituitaries from pregnant and virgin rats *in vitro* in response to CRH, since cAMP is a second messenger that is positively coupled to the CRH receptor in the pituitary (Antoni, 1986; King & Baertschi, 1990); we measured [^{125}I]CRH binding-site density in the pituitary to examine the possibility of CRH receptor downregulation. Finally, to see if there is an alteration in emotionality accompanying the attenuated HPA axis and HNS responses to stressors, the anxiety-related behaviour of pregnant and virgin rats on the elevated plus-maze (Pellow, Chopin, File & Briley, 1985) was monitored.

Preliminary results have been presented (Neumann, Johnstone, Landgraf, Russell & Douglas, 1996; Douglas, Johnstone, Hatzinger, Neumann, Landgraf & Russell, 1996).

METHODS

Animals

In experiment 1, virgin female Wistar rats (260–290 g) were mated overnight with sexually experienced males, and pregnancy was confirmed by the presence of a vaginal plug of semen in the mating cages the following morning (day 1 of pregnancy). Rats were housed in groups of four to six under standard laboratory conditions at the Max Planck Institute of Psychiatry (12:12 h light–dark cycle, lights on at 07.00 h, 22 °C, 60% humidity, food and water *ad libitum*) for at least 5 days before surgery and after delivery from the supplier (Charles River, Sulzfeld, Germany). Another group of virgin Wistar rats was mated overnight and housed singly 3 days prior to parturition; surgery was performed on day 4 to 6 of lactation. Lactating rats were kept with their litters until just before the experiments.

For experiments 2 and 3, virgin and pregnant Sprague–Dawley rats (Bantin and Kingman, UK), mated as above, were housed singly (12:12 h light–dark cycle, lights on at 07.00 h, 21 °C, food and water *ad libitum*) at the University of Edinburgh for at least 5 days prior to the experiment.

Experiment 1: ACTH, corticosterone, oxytocin and vasopressin secretory responses to emotional and physical stressors and to CRH in virgin, pregnant and lactating rats

Surgery for blood sampling

Under halothane (2–3%) anaesthesia and using sterile procedures, rats were implanted with chronic jugular vein catheters 5 days before the start of the experiments. The jugular vein was exposed

and a silicone tubing catheter (4 cm; Dow Corning) connected to a PE-50 polyethylene tubing was inserted approximately 3 cm into the vessel until the tip reached the right atrium; the catheter was exteriorized dorsally in the cervical region. The catheter was filled with sterile saline (0·9%) containing gentamicin (30 000 i.u. ml^{-1}; Centravet, Germany) and was flushed with the same solution after 3 days. Following surgery, rats were housed singly and handled carefully each day to familiarize them with the blood sampling procedure and to reduce non-specific stress responses during the experiments.

Behavioural testing and stress procedures

Elevated plus-maze. The elevated plus-maze has been validated for the detection of emotional responses to anxiogenic and anxiolytic substances (Pellow *et al.* 1985) and to stressful external stimuli (Heinrichs, Pich, Miczek, Britton & Koob, 1992; Liebsch *et al.* 1995). The plus-maze was used to assess the emotional state and as a mild emotional stressor (novel environment) in our experiment. The test is based on creating a conflict between the exploratory drive of the rat and its innate fear of open and exposed areas. Thus, increased open-arm exploration indicates reduced anxiety-related behaviour. As described in detail by Liebsch *et al.* (1995), the apparatus consists of a plus-shaped platform elevated 70 cm from the floor. Two of the opposing arms (50 cm × 10 cm) are closed by 40 cm-high side and end walls (closed arms), whereas the other two arms have no walls (open arms). At the beginning of the test, the rat was placed onto the central area (10 cm × 10 cm) of the maze. The following parameters were recorded by means of a video camera–computer set-up during the 5 min exposure: (1) entries into open arms (ratio of open-arm entries to total number of entries into all arms), (2) time spent on the open arms (ratio of time spent on open arms to total time spent on all arms), and (3) overall activity (total number of entries into closed arms).

Forced swimming. Forced swimming represents an ethologically relevant complex physical and emotional stressor for rats (Abel, 1994). With the extension tubing of the venous catheter still attached, rats were forced to swim for 90 s in a black plastic cylinder (40 cm in diameter and 50 cm in height) filled with tap water (19 °C) to a depth of *ca* 40 cm. After the swim, the rats were gently dried using towels for 10 s and returned to their home cages.

Experimental protocols

Stressors in virgin, pregnant and lactating rats (day 1). Five days after surgery, the responses of the HPA axis and the HNS to emotional and physical stressors were tested in virgin rats (289 ± 4·20 g; mean ± S.E.M.; $n = 19$), in rats on day 10 (327 ± 4·40 g; $n = 6$), day 15 (355 ± 7·30 g; $n = 7$), day 18 (374 ± 10·5 g; $n = 7$) and day 21 (430 ± 4·30 g; $n = 7$) of pregnancy and, in a separate experiment, in lactating rats (days 9–12 of lactation, 345 ± 5·80 g; $n = 7$) and their virgin controls (295 ± 5·90 g; $n = 5$).

At 08.00 h the catheters were attached to an extension tubing connected to a 1 ml plastic syringe filled with sterile heparinized saline (30 i.u. ml^{-1}), and the rats were left undisturbed for 90 min. Either 0·2 ml (for detection of ACTH and corticosterone) or 0·6 ml (for ACTH, corticosterone, oxytocin and vasopressin, and lactate as an indicator of muscle activity) blood samples, replaced immediately by sterile 0·9% saline, were taken under basal conditions at 09.30 and 10.00 h and after the respective stress exposure. For transfer to the elevated plus-maze, the catheter was disconnected and closed. Rats were then placed on the elevated plus-maze for 5 min, returned to their home cage and the catheter was reattached to the syringe. Ten minutes later, a blood sample was taken and immediately afterwards rats were exposed to the forced swim stress (90 s). Further blood samples were taken 5, 15 and 25 min after forced swimming.

At the end of the experiment, catheters were gently flushed with 0·3 ml gentamicin solution and closed.

Stimulation with I.V. CRH in virgin, pregnant and lactating rats (day 2). On the following day at 08.00 h, the rats were weighed, then the catheter was reconnected to a syringe filled with heparinized saline as described above, and at 09.30 and 10.00 h basal blood samples (0·2 ml for ACTH and corticosterone) were taken. Further blood samples replaced by sterile saline were collected 10, 30 and 50 min after I.V. bolus injection of human CRH (40 ng kg^{-1}, 80 ng ml^{-1}; Bissendorf Peptide, Wedemark, Germany).

At the end of the experiment, the rats were killed by an overdose of halothane and pregnancy state was carefully checked in all rats post mortem.

Treatment of blood samples

All blood samples were collected on ice in EDTA-coated tubes containing 10 μl aprotinin (Trasylol; Bayer AG) and centrifuged at 4 °C (5000 r.p.m., 5 min). Plasma samples were stored at −20 °C (80 μl for ACTH, 200 μl for oxytocin and vasopressin, 50 μl for lactate) or −80 °C (30 μl for corticosterone) until assay.

Radioimmunoassays (RIAs) for ACTH, corticosterone, oxytocin, vasopressin and plasma lactate measurement

Plasma ACTH and corticosterone concentrations were measured using commercially available kits (ICN) according to the respective protocols. The intra- and inter-assay coefficients of variation were below 7 and 10%, respectively. Plasma ACTH and corticosterone concentrations from virgin and pregnant, and virgin and lactating rats, respectively, were estimated in different assays.

Oxytocin and vasopressin concentrations were estimated in extracted plasma samples by highly sensitive and selective RIAs (detection limit, 0·1 pg $sample^{-1}$; cross-reactivity of the antisera with other related peptides, including vasopressin and oxytocin, was < 0·7%) (for a detailed description see Landgraf, 1981).

Plasma lactate concentrations were measured enzymatically (MPR1 Lactat; Boehringer Mannheim).

Experiment 2: *in vitro* basal and CRH-stimulated cAMP levels in pituitary segments from virgin and pregnant rats

Conscious virgin ($n = 16$) and day 11 ($n = 6$), day 17 ($n = 6$) and day 21 ($n = 8$) pregnant Sprague–Dawley rats caged separately were transferred individually to the experimental room immediately before decapitation between 09.00 and 11.00 h to minimize stress. Pituitaries were rapidly removed and washed in 0·5 ml Dulbecco's modified Eagle's medium (Gibco BRL), buffered with 25 mM Hepes to pH 7·4 and containing 0·25% bovine serum albumin (BSA; Sigma, RIA grade V) (thereafter solution referred to as DMEM). The posterior lobe was removed, anterior pituitaries weighed, and cut free-hand under microscopic control into eight similar segments. Pituitary segments were incubated for at least 1 h at 37 °C in 1 ml of DMEM in 24-well cluster plates (Costar). Subsequently, each segment was transferred into fresh DMEM (250 μl) containing 0·5 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). After incubation for 15 min at 37 °C, segments were challenged for a further 10 min with either 250 μl of 20 nM CRH (final concentration 10 nM; Peninsula, St Helens, UK) or vehicle (DMEM). The reaction was terminated by the addition of 0·5 ml ice-cold 0·2 N HCl, and culture plates were sealed with

parafilm and stored at −70 °C until trituration and cAMP determination.

Determination of cAMP

After two rounds of freeze–thawing (−70 to 0 °C), pituitary segments were homogenized by trituration through a gauge 26 needle. cAMP accumulation in duplicate samples of the acidic extract (pituitary tissue + extracellular medium) was determined using a specific double-antibody precipitation RIA as previously described (Woods, Shipston, Mullens & Antoni, 1992). Intra- and inter-assay variability was less than 5 and 10%, respectively.

Experiment 3: measurement of [^{125}I]CRH binding-site density in the pituitary gland

Virgin rats and rats on pregnancy day 11, 17 and 22 ($n = 6$ in each group) were housed singly for at least 1 week prior to tissue collection. Between 10.00 and 11.00 h the rats were decapitated and the whole pituitary carefully removed. Each pituitary was placed on a piece of aluminium foil on dry ice, covered in powdered dry ice and stored at −70 °C.

Horizontal pituitary cryostat sections (20 μm) were thaw-mounted onto poly-L-lysine-coated slides, desiccated overnight at 4 °C and stored at −20 °C in sealed boxes until use.

For receptor autoradiography, sections were preincubated for 30 min at 21 °C in 50 mM Tris–HCl (pH 7·4) containing 10 mM $MgCl_2$, 2 mM EGTA, 0·1% BSA (RIA grade V), 100 KIU ml^{-1} aprotinin (Trasylol), and 0·1 mM bacitracin (Sigma). Incubation was carried out subsequently in fresh buffer for 60 min with 0·2 nM [^{125}I]CRH (ovine; specific activity, 2200 Ci mmol^{-1}; DuPont). Non-specific binding was determined in the presence of 10^{-6} M unlabelled ovine CRH (Sigma). The sections were washed three times in BSA-free Tris–HCl buffer at 4 °C for 2 min, dipped in distilled water and rapidly dried in a stream of cold air. Dried sections were exposed to Agfascopix Video 5B films for 22 days.

Quantification of autoradiographs was performed microscopically (×10 objective lens; ×1·6 additional magnification) with a computer-assisted image-analysis system (Joyce-Loebl MicroMagiscan; Vickers, Gateshead, UK). Silver grain area per unit area (silver grain density) was measured within a 100 μm × 100 μm frame over each section. Total binding (silver grain density) was measured over two different areas per anterior pituitary section, one area for pars intermedia and one area for the posterior pituitary gland; for each animal such measurements were made on three pituitary sections. Similar measurements were made on two adjacent background areas off the tissue for each pituitary. Background silver grain density was subtracted from tissue grain density. For tissue incubated with excess cold CRH, three measurements per section of pituitary grain density were similarly made (pituitary lobes could not be distinguished). Mean density values were calculated for each rat per pituitary lobe, and the mean non-specific binding value subtracted from the total. Group means were then calculated.

Statistical analysis

Data are presented as group means ± S.E.M. To estimate total amount of hormone release in response to a given stimulus, the area under the curve corrected for baseline (AUC) was calculated using trapezoidal integration (Forsythe, Keenan, Organick & Stenberg, 1969). In addition, increments (δ) were calculated as the maximal poststimulation increase minus baseline values.

Statistical analysis was performed by means of statistical software (B-Stat V5.4, Dynamic Microsystems, USA, and SigmaStat, Jandel Scientific). The tests used were two-way (factors: pregnancy state × time) and one-way (factor: time) analyses of variance (ANOVA) for repeated measures followed by Newman–Keuls test (ACTH, corticosterone, oxytocin, vasopressin, lactate), or completely randomized one-way ANOVA (AUC, ratio, δ, plus-maze parameters during various pregnancy states, cAMP levels, CRH receptor autoradiography data) followed by Newman–Keuls test. $P < 0·05$ was considered statistically significant.

RESULTS

Experiment 1: neuroendocrine responses to emotional and physical stressors and to CRH in virgin, pregnant and lactating rats

Emotional and physical stressors (day 1)

ACTH and corticosterone. Basal plasma levels of ACTH and corticosterone at 09.30 or 10.00 h did not differ significantly between virgin rats and rats on day 10, 15, 18 or 21 of pregnancy ($P = 0·77$), although basal corticosterone levels in day 21 pregnant rats tended to be higher compared with all other groups (Fig. 1).

Mild emotional stress (5 min exposure to the elevated plus-maze) followed by a complex physical–emotional stressor (90 s forced swimming) significantly increased the secretion of ACTH and, consequently, of corticosterone in all groups ($P < 0·0001$; Fig. 1). The maximum rise in circulating hormone levels was reached 5 min (ACTH) and 15 min (corticosterone) after forced swimming. There was an inverse relationship between the stage of pregnancy and the magnitude of ACTH secretion in response to the elevated plus-maze with a *ca* 6·0- and 7·2-fold increase in virgin and day 10 pregnant rats, and a 3·1- and 1·9-fold increase in day 18 and day 21 pregnant rats, respectively. Similarly, subsequent forced swimming caused a 15- and 16-fold increase in plasma ACTH concentration in virgin and day 10 and only a 9·5- and 8·3-fold increase in day 18 and day 21 pregnant rats, respectively, compared with basal values ($P < 0·0001$; Fig. 1). Secretion of corticosterone in response to both the elevated plus-maze and forced swimming was also found to be significantly lower at the end of pregnancy ($P < 0·005$) with significant differences between virgin and day 10 pregnant rats, and day 18 and day 21 pregnant rats ($P < 0·01$; Fig. 1). The increasingly blunted neuroendocrine response to the emotional and physical stressors with the progression of pregnancy was also reflected by significantly reduced AUC values for ACTH (virgin, 17·1 ± 1·08 arbitrary units (a.u.); day 10, 18·8 ± 1·14 a.u.; day 15, 9·90 ± 1·45 a.u.; day 18, 9·50 ± 1·48 a.u.; day 21, 8·20 ± 3·90 a.u.; $P < 0·01$ days 15, 18 and 21 *vs.* day 10 and virgin); corresponding AUC values for corticosterone were not different between groups.

When the responses to exposure to emotional and physical stressors, respectively, throughout pregnancy were compared, as reflected by respective ACTH increments, the pregnancy-related difference in responsiveness was more pronounced during exposure to the elevated plus-maze, as there was a 6·2-fold difference in the increments between virgins and

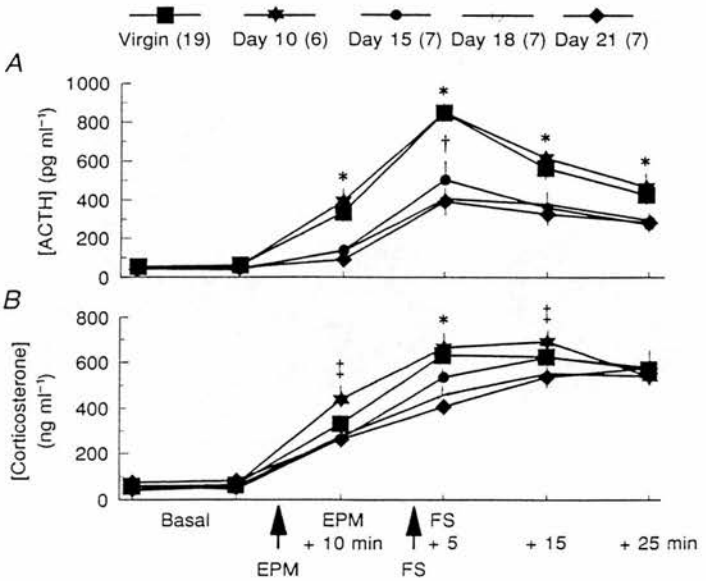

Figure 1

ACTH (*A*) and corresponding corticosterone (*B*) concentrations in plasma collected from the jugular vein of conscious virgin and day 10, 15, 18 and 21 pregnant rats on experimental day 1 under basal conditions (at 09.30 and 10.00 h) and 10 min after exposure to the elevated plus-maze (EPM; 5 min), as well as 5, 15 and 25 min after forced swimming (FS; 90 s, 19 °C). Five days before the experiment, rats were fitted with a jugular vein catheter under halothane anaesthesia and caged singly afterwards. Data are presented as means ± S.E.M.; numbers in parentheses indicate group size. Two-way ANOVA for repeated measures for ACTH (corticosterone); factor: time × group, $P < 0{\cdot}0001$ ($P < 0{\cdot}0083$). * $P < 0{\cdot}01$, virgin and day 10 *vs.* day 18 and 21; † $P < 0{\cdot}01$, day 15 *vs.* day 21; ‡ $P < 0{\cdot}01$, day 10 *vs.* day 18 and 21.

day 21 pregnant rats compared with a 1·7-fold difference after the forced swimming between these two groups ($P < 0{\cdot}05$). In a further series of experiments on Sprague–Dawley rats in Edinburgh, similar attenuation of ACTH and corticosterone secretion in response to swim stress alone was found in late pregnant rats (A. J. Douglas, H. Johnstone, A. Wigger, R. Landgraf, J. A. Russell & I. D. Neumann, unpublished data).

In lactating rats, the ACTH and corticosterone responses to both exposure to the elevated plus-maze and forced swimming were significantly less than those in virgin rats ($P < 0{\cdot}0001$; Fig. 2). Basal ACTH and corticosterone concentrations, respectively, were similar in both groups.

Oxytocin and vasopressin. Basal plasma levels of oxytocin differed significantly among virgin and pregnant rats (one-way ANOVA, $P < 0{\cdot}006$; Fig. 3), with increased basal oxytocin levels on days 18 and 21 compared with virgin and day 10 pregnant rats ($P < 0{\cdot}01$). The blood sample taken 15 min after forced swimming was used for the oxytocin and vasopressin assay. However, including pre- and poststimulation values of all groups, two-way ANOVA for repeated measurements did not reveal statistical differences among groups (factor: time × group; $P = 0{\cdot}532$). When pre- and poststimulation values were compared (factor: time; $P < 0{\cdot}0001$), forced swimming significantly increased the oxytocin concentration in the plasma only in virgin and day 10 and day 15 pregnant rats ($P < 0{\cdot}05$), but

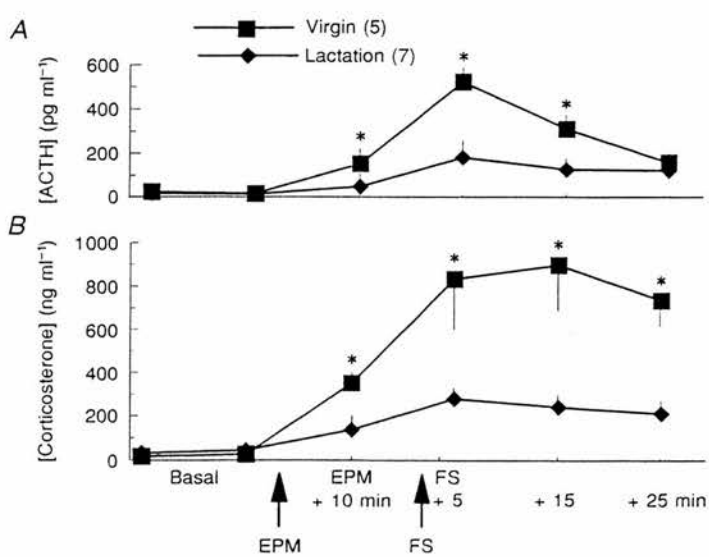

Figure 2

Plasma ACTH (*A*) and corticosterone (*B*) concentrations in virgin rats and between days 9 and 12 of lactation under basal conditions and in response to exposure to the elevated plus-maze (EPM) followed by forced swimming (FS) according to the protocol described in the legend to Fig. 1. Data are means ± S.E.M.; numbers in parentheses indicate group size. Two-way ANOVA for repeated measures for ACTH and corticosterone; factor: time × group, $P < 0{\cdot}0001$; * $P < 0{\cdot}01$, *vs.* lactating rats. Except for the ACTH level 10 min after EPM in lactating rats, ACTH and corticosterone concentrations were significantly increased at each time point after stimulation ($P < 0{\cdot}01$).

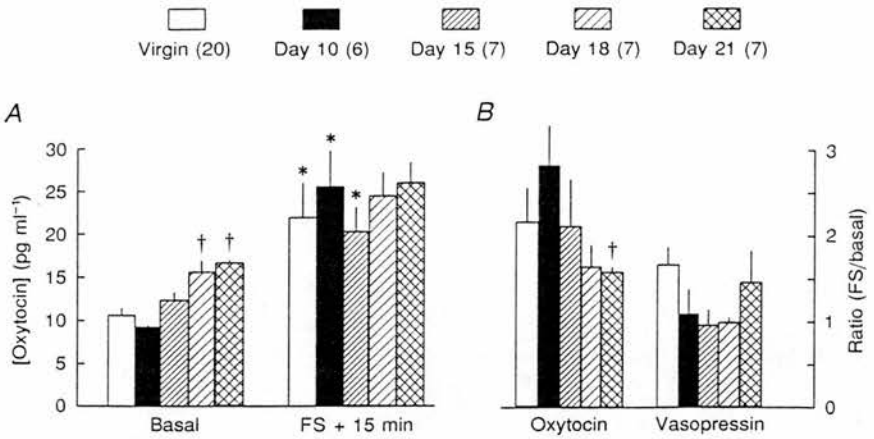

Figure 3

Plasma oxytocin concentration in conscious virgin and pregnant rats under basal conditions and 15 min after exposure to forced swimming (FS + 15 min) (*A*) as described in the legend to Fig. 1 and the calculated ratio between stimulated and basal neuropeptide concentrations for both oxytocin and vasopressin (*B*). Data are means ± S.E.M.; numbers in parentheses indicate group size. Two-way ANOVA for repeated measures; factor: time, $P < 0{\cdot}0001$; factor: group, $P < 0{\cdot}503$, factor: time × group, $P < 0{\cdot}7134$. * $P < 0{\cdot}05$, *vs.* corresponding basal values; † $P < 0{\cdot}045$, *vs.* virgin, day 10 (Kruskal–Wallis test, $P < 0{\cdot}0027$).

not in day 18 and 21 pregnant rats (Fig. 3). The reduced response to this kind of a combined emotional and physical stressor in late pregnancy is also reflected in a significantly lower ratio between poststimulation and prestimulation levels on day 21 compared with day 10 pregnant rats ($P < 0{\cdot}05$; Fig. 3).

In contrast to oxytocin, forced swim stress did not provoke an increase in vasopressin release into the blood, in either virgin or in pregnant rats (factor: time; $P = 0{\cdot}203$) as reflected by the respective ratio values (Fig. 3).

In lactating rats between days 9 and 12 of lactation, basal oxytocin and vasopressin levels did not differ in comparison with virgin rats (oxytocin: $7{\cdot}30 \pm 0{\cdot}50$ *vs.* $8{\cdot}05 \pm 0{\cdot}90$ pg ml^{-1}, $P = 0{\cdot}45$; vasopressin: $11{\cdot}1 \pm 2{\cdot}85$ *vs.* $12{\cdot}0 \pm 4{\cdot}15$ pg ml^{-1}). The stress-induced increase in oxytocin secretion was significantly reduced (lactating: 1·3-fold, virgin: 2·7-fold increase; $P < 0{\cdot}0002$) and did not reach statistical significance in the lactating group at any time point, i.e. 5, 15 or 50 min after the swim stress.

Again, plasma vasopressin levels remained unchanged in response to forced swimming in both virgin and lactating rats.

Lactate. In a separate follow-up study, when plasma lactate levels in day 21 pregnant and virgin rats both under basal conditions and in response to forced swimming were compared, plasma concentrations after stress were found to be significantly higher in late pregnant rats (from $8{\cdot}13 \pm 0{\cdot}74$ to $22{\cdot}7 \pm 3{\cdot}50$ mmol l^{-1}; virgin: from $6{\cdot}42 \pm 0{\cdot}82$ to

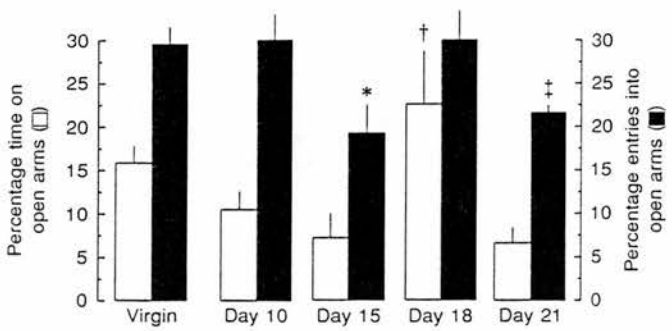

Figure 4

Anxiety-related behaviour of virgin and day 10, 15, 18 and 21 pregnant rats on the elevated plus-maze on day 1 of the experiment as indicated by the percentage of time spent on the open arms (□) and by the percentage of entries into the open arms (■). For details see legend to Fig. 1. One-way ANOVA; percentage time: $P < 0{\cdot}021$; † $P < 0{\cdot}01$, *vs.* day 15 and 21. One-way ANOVA; percentage entries: $P < 0{\cdot}027$; ‡ $P < 0{\cdot}05$, *vs.* day 10, * $P < 0{\cdot}01$, *vs.* virgin, day 10 and day 18. Data are means ± S.E.M.

Table 1. Total cyclic AMP content in segments of the adenohypophysis of day 11, day 17 and day 21 pregnant rats and their respective virgin controls incubated either in vehicle (Basal) or CRH (10 nM) at 37 °C for 10 min, and the respective increments (δ; CRH-stimulated minus vehicle)

	[cAMP] (nmol)		
	Basal	CRH-stimulated	δ
Day 11 (6)	7·06 ± 0·76	13·4 ± 1·28	6·92 ± 1·15
Virgin (4)	6·90 ± 1·60	14·3 ± 3·48	7·72 ± 2·04
Day 17 (6)	6·10 ± 0·78*	15·3 ± 0·83*	9·22 ± 0·68
Virgin (4)	10·4 ± 1·31	18·8 ± 1·81	8·38 ± 1·42
Day 21 (8)	5·20 ± 0·27*	11·5 ± 0·70*	6·30 ± 0·68**
Virgin (8)	7·06 ± 0·89	16·3 ± 1·38	9·26 ± 0·86

Data are means ± S.E.M.; numbers in parentheses indicate group size. ** $P < 0·01$, * $P < 0·05$, *vs.* respective virgin group.

14·7 ± 2·29 mmol l^{-1}; $n = 7$ each; $P < 0·01$) with no significant differences in basal levels.

Anxiety-related behaviour on the elevated plus-maze. With respect to their anxiety-related behaviour, virgin rats and rats at different stages of pregnancy differed significantly ($P < 0·021$; Fig. 4). In general, pregnant rats evidently became more anxious with the progression of pregnancy, i.e. on days 15 and 21, compared with virgin and day 10 pregnant animals, as indicated by a reduced percentage of entries into the open arms ($P < 0·05$; Fig. 4) as well as the tendency for a reduced percentage of time spent on the open arms during the 5 min exposure on the plus-maze. On day 18 only, pregnant rats spent significantly more time on the open arms and thus seemed, by this measure, to be less anxious compared with day 15 and day 21 pregnant animals ($P < 0·01$; Fig. 4). The locomotor activity, reflected by the total number of entries into the closed arms, did not differ among groups ($P = 0·57$).

Lactating and virgin rats did not differ in their anxiety-related behaviour (16·3 ± 3·17 *vs.* 13·8 ± 2·29% of total time spent in open arms, respectively).

Intravenous administration of CRH (day 2)

ACTH and corticosterone. On day 2 of the experiment, basal levels of circulating corticosterone, but not ACTH, between 09.00 and 10.00 h differed significantly among groups ($P < 0·0001$), with elevated corticosterone levels on day 22 of pregnancy compared with virgins and rats on days 11, 16 and 19 of pregnancy ($P < 0·01$; Fig. 5).

Intravenous administration of CRH (40 ng kg^{-1}) increased ACTH and, consequently, corticosterone secretion in all groups studied (factor: time; $P < 0·0001$ for both). Maximum

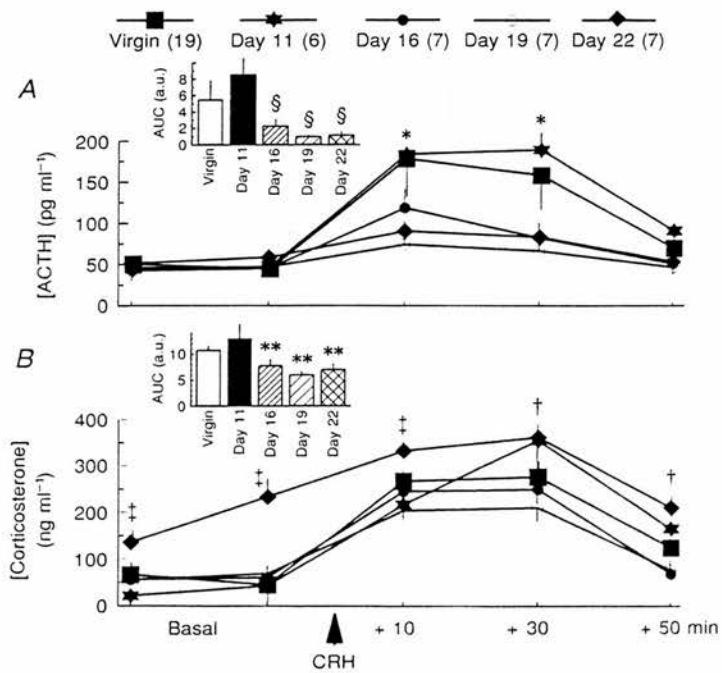

Figure 5

ACTH (*A*) and corresponding corticosterone (*B*) concentrations in plasma collected from the jugular vein of conscious virgin and day 11, 16, 19 and 22 pregnant rats on experimental day 2 in response to I.V. CRH (40 ng kg^{-1}, arrow). For details see legend to Fig. 1. Insets show the area under the curve corrected for baseline (AUC; in arbitrary units (a.u.)). Data are means ± S.E.M.; numbers in parentheses indicate group size. * $P < 0·01$, virgin and pregnancy day 11 *vs.* days 19 and 22; † $P < 0·05$, pregnancy day 11 *vs.* days 16 and 19; ‡ $P < 0·01$, pregnancy day 22 *vs.* all other groups; § $P < 0·01$, ** $P < 0·05$, *vs.* virgin and day 11.

hormone levels were reached 10 min (ACTH) and 30 min (corticosterone) after administration of CRH. CRH was less effective with the progression of pregnancy (factor: time × group; $P < 0{\cdot}05$ for both) with significant differences in ACTH secretion at 10 min among virgin (4·4-fold increase), day 11 (4·0-fold) and day 16 (2·6-fold), day 19 (1·6-fold) and day 22 (1·5-fold) pregnant rats. This was also reflected by reduced AUC values for both ACTH and corticosterone on days 16, 19 and 22 of pregnancy (Fig. 5).

In lactating rats, basal ACTH levels tended to be higher compared with virgin rats ($18{\cdot}2 \pm 3{\cdot}71$ *vs.* $8{\cdot}76 \pm 1{\cdot}78$ pg ml^{-1}, n.s.). There was a significant difference between lactating and virgin rats in their response to i.v. CRH ($P < 0{\cdot}001$) with no significant response in lactating rats and a 2·4-fold increase ($P < 0{\cdot}01$) in virgin rats at 10 min.

Experiment 2: *in vitro* basal and CRH-stimulated cAMP levels in pituitary segments from virgin and pregnant rats

In the presence of the phosphodiesterase inhibitor IBMX, basal as well as CRH-stimulated cAMP accumulation was significantly lower in pituitary segments isolated from day 17 and day 21 pregnant rats compared with the respective virgin groups (Table 1), whereas no such difference was found in pituitary segments from day 11 pregnant rats compared with their virgin controls. Importantly, the CRH-induced increment (CRH-stimulated minus basal) in cAMP accumulation was significantly attenuated in late pregnant rats on day 21 of pregnancy ($P < 0{\cdot}05$; Table 1) indicating a reduced efficacy of CRH-stimulated accumulation compared with virgin controls.

There was no difference (ANOVA, $P < 0{\cdot}066$) in anterior pituitary weights between virgin ($11{\cdot}1 \pm 0{\cdot}2$ mg), day 11 ($11{\cdot}1 \pm 0{\cdot}14$ mg), day 17 ($11{\cdot}6 \pm 0{\cdot}37$ mg) and day 21 ($12{\cdot}1 \pm 0{\cdot}35$ mg) pregnant rats.

Experiment 3: measurement of [^{125}I]CRH binding-site density in the pituitary gland

[^{125}I]CRH binding-site density as reflected by silver grain densities was estimated in the anterior, posterior and intermediate pituitary lobes of virgin and day 11, 17 and 22 pregnant rats. Compared with virgin rats, there was a significantly reduced [^{125}I]CRH binding in the anterior pituitary of all pregnancy groups studied ($P < 0{\cdot}01$ *vs.* virgin; Fig. 6) and a further reduction in specific [^{125}I]CRH binding on day 22 compared with days 11 and 17 of pregnancy ($P < 0{\cdot}05$; Fig. 6). [^{125}I]CRH binding in the intermediate lobe of the pituitary was detectable and was found to be reduced in day 22 pregnant rats ($P < 0{\cdot}05$ *vs.* virgin). In the posterior pituitary, virtually no specific [^{125}I]CRH binding was detectable.

DISCUSSION

The present results demonstrate that, in rats, the attenuated HPA axis responsiveness to stressors observed during lactation is already manifested in mid-gestation. Specifically, we showed a significant hyporesponsiveness to both emotional (elevated plus-maze) and physical (forced swimming) stressors from day 15 until day 21 of pregnancy as well as during lactation, reflected in a reduced stress-induced secretion of ACTH from the adenohypophysis and reduced corticosterone secretion from the adrenal gland. Similarly, the oxytocin secretory response to forced swimming tended to be reduced on the last days of pregnancy. The attenuated response of the HPA axis is likely to be at least partly due to a reduced reactivity of corticotrophs to CRH as demonstrated *in vivo* by reduced CRH-stimulated ACTH secretion from the adenohypophysis into blood and *in vitro* by lower stimulation by CRH of cAMP production in corticotrophs of the adenohypophysis. Moreover, receptor autoradiography revealed a significant reduction in [^{125}I]CRH binding-site density in the anterior pituitary of pregnant (days 11, 17 and 22) compared with virgin rats. Independent of the reproductive state, the neuroendocrine responses to the emotional stressor did not correlate with the anxiety-related behaviour of the animals on the elevated plus-maze.

Basal and stress-induced activity of the HPA axis and HNS during pregnancy

In our study, basal (a.m.) levels of ACTH remained stable throughout pregnancy, including on day 22; these findings are comparable with the trough ACTH values in pregnant rats described by Atkinson & Waddell (1995). In human and ovine pregnancy, increased basal ACTH concentrations may be partly placental in origin (for review, see Keller-Wood,

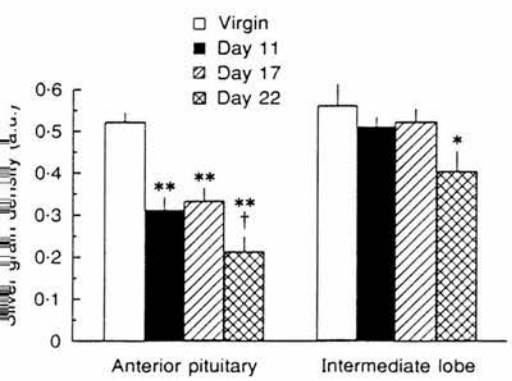

Figure 6
Specific [^{125}I]CRH binding in the anterior and intermediate lobe of pituitaries collected from virgin, day 11, day 17 and day 22 pregnant rats ($n = 6$ each). The film was quantified by image analysis to measure silver grain density (arbitrary units; a.u.). Data are means ± S.E.M. * $P < 0{\cdot}05$ and ** $P < 0{\cdot}01$, *vs.* virgin; † $P < 0{\cdot}05$, *vs.* day 11 and day 17 pregnant groups.

1994). In the rat, the placenta may be a source of circulating ACTH (Chen, Chang, Krieger & Bardin, 1986), although this is uncertain (Waddell, 1993), but this is not released in response to maternal stress (Ohkawa, Takeshita, Murase, Kambegawa, Okinaga & Arai, 1991). Similarly, basal corticosterone concentrations did not differ between virgin and pregnant rats, except for an increase on day 22 of pregnancy, the first expected day of parturition. Previously, decreased plasma corticosterone concentration has been found early in pregnancy (Ogle & Kitay, 1977; Atkinson & Waddell, 1995) followed by a return to prepregnancy levels and a further increase on the last day(s) of pregnancy as we found. This late increase in plasma corticosterone concentration may reflect increased sensitivity of the adrenal glands to ACTH, reduced metabolic clearance rate (Waddell & Atkinson, 1994), stimulation by increased ovarian oestrogen secretion or a contribution by the fetal adrenal glands (Dupont, Rheaume, Simard, Luu-The, Labrie & Pelletier, 1991).

Our novel finding is that the response of the HPA axis to both emotional and physical stressors is significantly blunted in the pregnant rat, beginning around day 15. These adaptations of the HPA axis manifest in mid-gestation seem to persist throughout lactation thus confirming and extending recent results (Altemus *et al.* 1995; Walker *et al.* 1995; Neumann *et al.* 1995*b*; da Costa, Wood, Ingram & Lightman, 1996; Windle *et al.* 1997). However, the mechanisms of adaptation may not be the same in pregnancy and lactation. Diminished ACTH secretion in response to stimuli including CRH in pregnancy has been described also in sheep (Keller-Wood, 1994), baboons (Goland, Wardlaw, MacCarter, Warren & Stark, 1991) and humans (Magiakou, Mastorakos, Rabin, Dubbert, Gold & Chroussos, 1996).

The pregnancy-related adaptations of the stress response include the HNS with oxytocin being released in response to a variety of stressors in the rat (Lang *et al.* 1983; Kasting, 1988; Wotjak *et al.* 1996). There are increased stores of oxytocin in the neural lobe (Douglas, Dye, Leng, Russell & Bicknell, 1993) and basal oxytocin secretion increases toward the end of pregnancy as confirmed in the present study (Fig. 3). However, the stress-induced rise in oxytocin release in late pregnancy was slightly attenuated and did not reach statistical significance in day 18 and 21 pregnant rats; restraint by endogenous opioid peptides may be important (Douglas *et al.* 1995). Similarly there is an increased basal and attenuated stress-induced local release of oxytocin within the hypothalamic paraventricular nucleus (PVN) at the end of pregnancy (Neumann *et al.* 1997). The altered oxytocin responses in pregnant rats differ from the pattern of attenuated responses of the HPA axis, which were markedly reduced from day 15 of pregnancy onward, through lactation (Figs 1 and 2). In lactation, the oxytocin response was essentially abolished, consistent with previous reports of reduced responses to non-suckling-related stimuli (Carter & Lightman, 1987; Lightman & Young, 1989; Patel *et al.* 1991; Koehler *et al.* 1993; Neumann *et al.* 1995*a*, *b*).

Mechanisms of the attenuated HPA axis activity during pregnancy

The physiological adaptations of the HPA axis during gestation occur at several levels including limbic feedback systems (Johnstone, Douglas, Seckl & Russell, 1997), CRH/vasopressin neurons within the hypothalamus (Douglas & Russell, 1994), corticotrophs of the adenohypophysis as shown in the present study, and cortical cells of the adrenal gland (Carr *et al.* 1981; Waddell & Atkinson, 1994). An enhanced glucocorticoid feedback especially at hippocampal, but also hypothalamic, brain areas would negatively control CRH/vasopressin neurons and, thus, attenuate ACTH secretion. With respect to glucocorticoid feedback control during pregnancy, various species-dependent results have been published with unchanged (Keller-Wood, 1996) or reduced feedback sensitivity (Owens *et al.* 1987). Changes in glucocorticoid receptor binding capacity within the hippocampus as observed in lactation (Meaney, Viau, Aitken & Bhatnagar, 1989) are being studied in our laboratories also during pregnancy (Johnstone *et al.* 1997). However, the stress response is also reduced in adrenalectomized lactating rats (Walker *et al.* 1992), so altered feedforward control mechanisms of the HPA axis could be involved. The pregnancy-related alterations in plasma levels of the sex steroids, oestrogens and progesterone, could alter hippocampal mineralo- (Carey, Deterd, de Koning, Helmerhorst & de Kloet, 1995) and glucocorticoid (Burgess & Handa, 1992) receptor binding, hypothalamic CRH expression (Douglas & Russell, 1994; Grino, Héry, Paulmyer-Lacroix & Anglade, 1995) and ACTH and corticosterone secretion (Viau & Meaney, 1991; Burgess & Handa, 1992). However, it is uncertain whether the secretion of hypothalamic corticotrophin-releasing factors is reduced during pregnancy (Plotsky, 1986).

In the present study several lines of evidence indicate pregnancy-related alterations at corticotrophs of the pituitary synthesizing and releasing ACTH. In rats between days 16 and 22 of pregnancy, these cells showed a reduced response to CRH *in vivo* as reflected in a reduced ACTH secretion (Fig. 5). Although receptor autoradiography revealed a reduction in CRH receptor density in pregnant rats beginning on day 11 no significant reduction of CRH-stimulated cAMP accumulation was observed until day 17 of pregnancy. The normal CRH response, in terms of cAMP accumulation *in vitro*, observed between days 10 and 16 in the face of a marked reduction in CRH receptor density is most probably a result of the large CRH receptor capacity reported in corticotrophs (Antoni, 1986; King & Baertschi, 1990). This suggests that the reduced CRH-stimulated ACTH response observed *in vivo* at days 11–17 of pregnancy is not a consequence of alterations in CRH receptor coupling to the cAMP pathway but results from alterations either downstream of cAMP accumulation in

corticotrophs or as a result of pregnancy-induced changes at higher levels of the HPA axis. The reduced CRH-stimulated cAMP accumulation at day 21 of pregnancy, perhaps resulting from a further decrease in CRH receptor density, may underly the mechanisms of attenuated ACTH responses *in vivo* at this time. However, it should be noted that, in the rat, the placenta does not synthesize CRH as found in several other mammalian species (Jones, Gu & Parer, 1989; Robinson, Arbiser, Emanuel & Majzoub, 1989; Goland *et al.* 1991); thus, the downregulation of pituitary receptors is not likely to be due to an increased level of circulating CRH of peripheral origin.

We can exclude the possibility that reduced locomotor activity during exposure to the elevated plus-maze or forced swimming led to reduced HPA axis responses in pregnant rats, since (i) the total number of entries into the closed arms of the plus-maze, and (ii) the amount of time that animals spent struggling, swimming or floating during forced swimming were similar among groups. The increase in plasma lactate concentration, indicative of muscle activity during the swim stress (Abel, 1994), was even higher in late pregnant rats. Alternatively, reduced stressor perception, resulting from lowered stimulatory inputs from suprahypothalamic, e.g. limbic and cortical, brain areas and the brainstem to the hypothalamic PVN containing CRH/vasopressin neurons could be postulated; consistent with this is reduced stress-induced c-fos mRNA expression within the parvocellular part of the PVN in late pregnant and lactating rats (da Costa *et al.* 1996). Although our experimental design did not allow a clear distinction between emotional and physical components, our observations suggest that the pregnancy-related reduction in ACTH secretion is at least as pronounced in response to a mild emotional as it is to a predominantly physical stressor (Fig. 1), thus supporting the notion of reduced stress perception.

Significance of reduced HPA axis and HNS responsiveness during pregnancy

Negative effects of exogenous ACTH on implantation and the progress of gestation were described and interpreted as an excessive stimulation of secretion of steroids other than corticosteroids from the adrenal gland (Chatterjee & Harper, 1970). Daily treatment with ACTH during the last third of gestation causes abnormal development of the young, prolonged pregnancy and impairs the onset of maternal behaviour (Fameli, Kitraki & Stylianopoulou, 1993). Similarly, increased HPA axis activity during pregnancy triggered by excessive stress adversely affects the behavioural (Fameli, Kitraki & Stylianopoulou, 1994) and endocrine development of the offspring (for review see Weinstock, 1997). Comparable findings were observed in the offspring of aged pregnant females with impaired glucocorticoid feedback and thus elevated corticosteroid levels during pregnancy (Erisman, Carnes, Takahashi & Lent, 1990). Thus, the dampened stress response of the maternal HPA axis during normal pregnancy described in the present study may provide a protective mechanism against excessive levels of circulating ACTH/corticosteroids during the sensitive period of fetal HPA axis development and maturation.

In contrast, the attenuated stress-induced release of oxytocin at the last day(s) of pregnancy may serve the purpose of storing neurohypophysial oxytocin prior to delivery when demands for neurohypophysial oxytocin are high.

Dissociation between neuroendocrine and behavioural responses throughout pregnancy

The neuroendocrine responses to the emotional stressor were not correlated with the anxiety-related behaviour of virgin, pregnant or lactating rats. Thus, despite a continuous reduction in the HPA axis responsiveness with the progression of pregnancy, pregnant rats (except on day 18 of pregnancy) were evidently more anxious on the elevated plus-maze compared with virgin controls. Interestingly, in contrast to pregnant animals, lactating rats tended to be less anxious on the elevated plus-maze. A reduced anxiety in lactating rats has been shown by studying the duration of freezing in response to an auditory stimulus (Hard & Hansen, 1984). These behavioural alterations, however, might be related to the complex patterns of maternal behaviour which include an increased aggressive behaviour towards conspecifics (Erskine, Barfield & Goldman, 1978). Generally, in both pregnant and lactating rats, the results indicate an independent regulation of HPA axis activity and anxiety-related behaviour thus confirming recent results (Pich, Heinrichs, Rivier, Miczek, Fisher & Koob, 1993; Walker *et al.* 1995).

In conclusion, we have demonstrated reduced responsiveness of the HPA axis to various stressors in pregnancy. This involves reduced corticotroph responsiveness to CRH with reduced CRH receptor density at the adenohypophysis. This adaptation may protect the fetuses from lifelong adverse effects of exposure to excessive glucocorticoids.

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The role of endogenous opioids in neurohypophysial and hypothalamo–pituitary–adrenal axis hormone secretory responses to stress in pregnant rats

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Abstract

Endogenous opioid regulation of neurohypophysial and hypothalamo–pituitary–adrenal (HPA) axis hormone secretion in response to forced swimming (90 s in deep water at 19 °C) was investigated in virgin and 21-day-pregnant rats. There was no difference in basal plasma oxytocin concentrations between pregnant and virgin rats, but the opioid antagonist, naloxone, increased basal oxytocin secretion in the pregnant rats. Forced swimming increased oxytocin secretion similarly in pregnant and virgin rats, and this response was enhanced by naloxone. In pregnant rats naloxone had a greater effect (by 3·1-fold) than in virgins, showing stronger endogenous opioid restraint of an enhanced oxytocin secretory response to stress in pregnancy. Vasopressin secretion was not increased with forced swimming in virgin or pregnant rats, and naloxone had no effect. ACTH and corticosterone secretion in response to forced swimming was attenuated in pregnant rats compared to virgin rats, measured at 5 min. Naloxone had no effect on basal plasma ACTH or corticosterone concentration, but it reduced ACTH secretion in virgin rats 5 min after forced swimming; in pregnant rats naloxone had no such effect. Naloxone removed the pregnancy-related attenuation in corticosterone secretion measured at 5 min after forced swimming. Fifteen minutes after forced swimming, plasma corticosterone concentrations were not different between groups. In the late-pregnant rats, the increases in plasma ACTH and corticosterone induced by forced swimming were significantly prolonged compared to virgins. The results show that endogenous opioid inhibition emerges in pregnancy to restrict the responses of oxytocin neurones to a stressor. In contrast, the endogenous opioid enhancement of mechanisms regulating HPA axis secretory responses in virgin rats is not evident during pregnancy.

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Introduction

In addition to the well-known stimulation of hypothalamo–pituitary–adrenal (HPA) axis hormone secretion by a wide variety of stressors, there are robust neurohypophysial secretory responses to stressors; for example, forced swimming, immobilisation, social defeat and intraperitoneal hypertonic saline induce increased release of oxytocin into the blood (Lang *et al.* 1983, Gibbs 1986, Wotjak *et al.* 1996), whereas ether, haemorrhage, hypoxia and noxious stimuli increase the release of vasopressin (Gibbs 1986, Yagi 1992). Oxytocin secretion in response to a stressor is restrained by endogenous opioids in female, but not in male, rats (Carter *et al.* 1986, Carter & Lightman 1987*a*), and opioids also influence HPA axis activity (Buckingham & Cooper 1984, Plotsky 1986, Suda *et al.* 1992, Calogero 1996). Both oxytocin and HPA axis stress responses are reduced in lactation (Carter & Lightman 1987*b*, Higuchi *et al.* 1988, Walker *et al.* 1995, Windle *et al.* 1997). We have recently shown that the HPA axis response to stress is attenuated in pregnancy from day 15 onward (Neumann *et al.* 1998) as well as in lactation (e.g. Walker *et al.* 1995, Windle *et al.* 1997). Although pituitary mechanisms account for part of these inhibited secretory responses (Johnstone *et al.* 1997, Neumann *et al.* 1998), central, hypothalamic mechanisms are also likely to play a part, as parvocellular paraventricular nucleus (PVN) neurone expression of Fos in response to stress is attenuated in pregnancy (da Costa *et al.* 1996). Because stimulation of oxytocin neurone activity and secretion by the brainstem input are restrained by endogenous opioids in late pregnancy (Douglas *et al.* 1995), and secretion of another stress hormone, prolactin, is also inhibited by opioids in pregnancy (Soaje & Deis 1994), we hypothesised that there may be a common, central opioid mechanism inhibiting neuroendocrine stress responses in pregnancy.

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We have now investigated whether endogenous opioids modify oxytocin secretion in response to a stressor in pregnancy. We have also compared the secretion of oxytocin with vasopressin secretion. In addition, we have sought a role for endogenous opioids in restraining the HPA axis secretory responses in pregnancy.

Material and Methods

Animals

Virgin female Sprague–Dawley rats (260–290 g body weight) were mated overnight with sexually experienced males and pregnancy was confirmed by finding a vaginal plug of semen (day 1 of pregnancy). Rats were housed singly under standard laboratory conditions (12 h light : 12 h darkness cycle, lights on at 0700 h, 22 °C, 60% humidity, food and water available *ad libitum*) for at least 7 days before surgery.

Surgery Under halothane anaesthesia and with sterile procedures, rats were implanted with a chronic jugular vein catheter (silastic tubing inside diameter 0·5 mm, outside diameter 0·75 mm, Altec, Alton, UK) 3–4 days before the experiment. The catheter was filled with heparinised (20 IU/ml, Multiparin, CP Pharmaceuticals Ltd, Wrexham, UK) sterile isotonic (0·9% w/v) saline. After surgery, the rats were housed singly and familiarised to daily handling.

Effect of naloxone on secretory responses to forced swimming in pregnant rats

Three to four days after surgery, the effect of naloxone (a general opioid antagonist) on neurohypophysial and HPA axis hormone secretory responses to forced swimming was tested. Blood samples were taken before and after treatment with naloxone or vehicle in virgin (n=7, body weight 282 ± 6 g; n=8, body weight 286 ± 9 g respectively) and 21-day-pregnant rats (n=7, 364 ± 9 g; n=8, 362 ± 9 g respectively), and subsequently after a period of forced swimming.

At 0800 h, the catheter was attached to an extension tubing (polythene, outside diameter 1·0 mm) connected to a syringe filled with sterile heparinised saline (20 IU/ml), and the rats were left undisturbed for 90 min. Blood samples (0·65 ml), substituted immediately by sterile 0·9% saline, were taken under basal conditions at 0930 h and 1000 h. Then naloxone (5 mg/kg, 50 µl/100 g body weight) or vehicle was injected i.v. and a further sample taken 15 min later. After an interval of 20–30 min, rats were exposed to forced swimming, a combined physical and emotional stressor (Abel 1994). With the extension tubing of the venous catheter still attached, rats were forced to swim for 90 s in a bucket filled with tap water (19 °C) to a depth of about 40 cm. After the swim, the rats were gently dried, using towels, for 10 s and returned to their home cages. Further blood samples were taken 5, 15 and 60 min after forced swimming. All blood samples were collected on ice in tubes containing EDTA (5% solution, 15 µl/100 µl blood) supplemented with aprotinin (0·039 trypsin inhibitor units/tube; Sigma, Poole, Dorset, UK) and centrifuged. Plasma samples (200 µl for oxytocin and vasopressin, 80 µl for ACTH and 50 µl for corticosterone) were stored at − 20 °C until required for assay.

Hormone assays

Oxytocin and vasopressin concentrations were measured in extracted plasma samples by highly sensitive and selective radioimmunoassays (limit of detection 0·1 pg/sample; cross-reactivity of the antisera with other related peptides, including oxytocin or vasopressin, <0·7%; for a detailed description see Landgraf 1981).

Plasma ACTH was measured by radioimmunoassay using a commercially available kit (ICN, Costa Mesa, CA, USA). The intra- and interassay coefficients of variation were less than 7 and 10% respectively. Total plasma corticosterone was measured by radioimmunoassay using a scintillation proximity method. Briefly, plasma samples were denatured by incubation in borate buffer (133 mM boric acid, 68 mM NaOH; pH 7·4, 1 : 9 v/v) containing bovine serum albumin (0·5%) in a 96 well microtitre plate (Falcon) at 80 °C for 30 min. Then the samples and a range of standards were incubated with ^{3}H-corticosterone (Amersham Life Sciences, Little Chalfont, Bucks, UK; 11 000 c.p.m. per well) and anti-corticosterone antibody (1 : 10 000 dilution, rabbit anti-rat, a gift from the High Blood Pressure Unit, Western General Hospital, Glasgow, UK) in a total volume of 70 µl for 1 h at room temperature. Scintillation proximity assay reagent (anti-rabbit, Amersham Life Sciences, 50 µl, which holds antibody-bound radioactivity in close proximity to scintillant) was mixed in and incubated for a further 24 h at room temperature before counting in a β-scintillation counter. The intra-assay coefficient of variation was 6%.

Statistical analysis

Statistical analysis was performed by means of statistical software (Sigmastat, Jandel Scientific, Erkrath, Germany). Data are presented as group means ± S.E.M. Because the data were from four groups, representing two reproductive states, and each set contained repeated measurements from the same animal, two-way analysis of variance (ANOVA, reproductive state × time) for repeated measures followed by Newman–Keuls *post hoc* test was used to compare the interactions between all data. One-way ANOVA was also utilized to compare basal values and calculated increments in secretory responses. To analyse

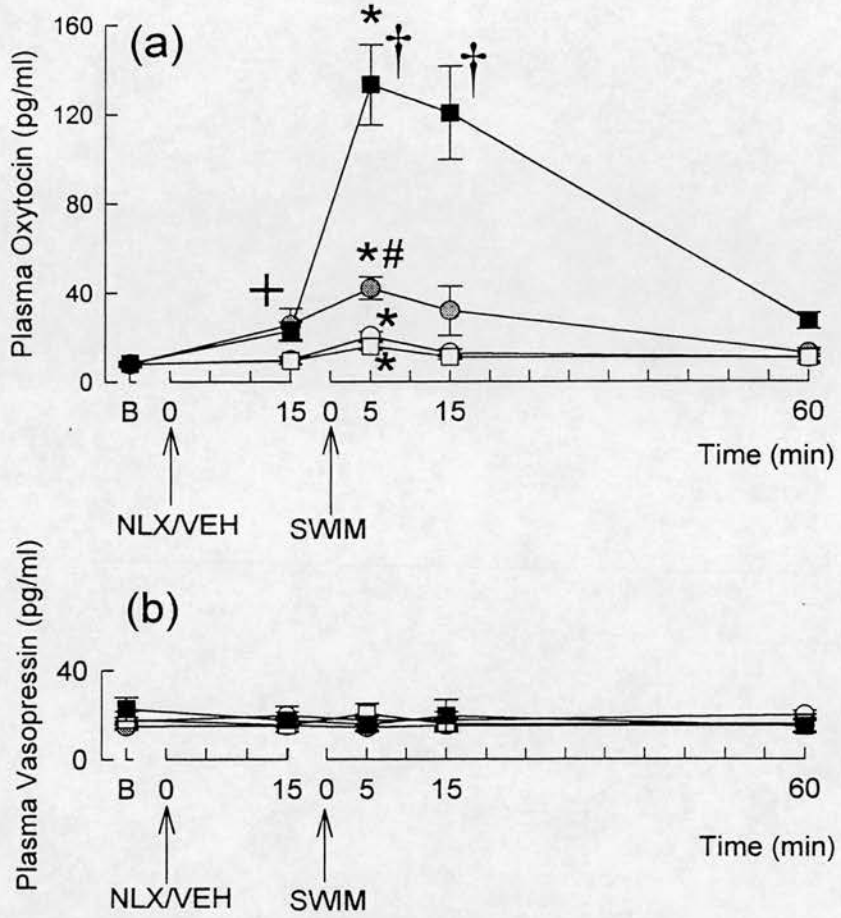

Figure 1 Effect of naloxone on neurohypophysial hormone secretory responses to forced swimming in pregnancy. Plasma oxytocin (a) and vasopressin (b) concentrations in virgin, vehicle-treated (VEH, ○, n=7) and naloxone-treated (NLX, ◍, n=8) rats, and in 21-day-pregnant vehicle-treated (□, n=7) and naloxone-treated (■, n=8) rats. Newman-Keuls *post hoc* tests, *$P<0{\cdot}05$ compared with before swim in all groups; †$P<0{\cdot}05$ compared with all other groups at same time point. +$P<0{\cdot}05$ pregnant naloxone-treated group compared with pregnant vehicle-treated group and compared with before injection ($P<0{\cdot}01$ one-way ANOVA for repeated measures). #$P<0{\cdot}01$, *t*-test, increment in plasma oxytocin above basal in virgin naloxone-treated group compared with virgin vehicle-treated group.

specifically the different responses to stress after naloxone compared with those after vehicle in the pregnant and virgin rats, the standard error of the difference between the means (vehicle- and naloxone-treated) 5 min after forced swimming was used (*t*-test, Swinscow 1983). $P<0{\cdot}05$ was considered statistically significant.

Results

Effect of naloxone on neurohypophysial hormone secretory responses to forced swimming in pregnancy

Two-way ANOVA for repeated measures of the plasma oxytocin concentrations in all groups showed a significant difference between groups ($P<0{\cdot}001$) and across time ($P<0{\cdot}001$); all other specific comparisons were derived from *post hoc* tests ($P<0{\cdot}05$), unless stated otherwise. Basal plasma oxytocin concentration was not significantly different between virgin and pregnant rats, and after forced swimming, plasma oxytocin concentration increased significantly in all groups within 5 min after the swim compared with values before the swim (Fig. 1a); there was no significant difference in the response between the vehicle-treated virgin and pregnant rats. Within 15 min after the forced swimming, the plasma oxytocin concentration had returned to pre-stress values in the virgin and vehicle-treated pregnant rats. Naloxone increased basal plasma oxytocin significantly only in pregnant rats (one-way ANOVA for repeated measures, $P<0{\cdot}05$, Fig. 1a). Naloxone also significantly enhanced the oxytocin

secretory response to forced swimming in both virgin (5·1-fold greater than basal concentration, compared with 2·6-fold greater in vehicle-treated virgin rats; $P<0·01$, *t*-test, Fig. 1a) and pregnant rats (15·9-fold greater than basal concentration, compared with 1·9-fold greater than basal in vehicle-treated pregnant rats; $P<0·0001$, *t*-test, Fig. 1a); the effect of naloxone in pregnancy was 3·1-fold greater than in virgins. Plasma oxytocin concentration remained increased in the naloxone-treated pregnant rats 15 min after the swim, but returned to the concentration recorded before the swim in the naloxone-treated virgin rats.

Basal plasma vasopressin concentration was not significantly different between pregnant and virgin groups, and did not alter after naloxone or with forced swimming in any group (Fig. 1b).

Effect of naloxone on HPA axis secretory responses to forced swimming in pregnant rats

ACTH Two-way ANOVA for repeated measures of the plasma ACTH concentrations in all groups showed a significant interaction between time and group ($P<0·001$); all other specific comparisons were derived from *post hoc* tests ($P<0·05$), unless stated otherwise. Basal plasma concentrations of ACTH did not differ significantly between virgin and day 21 pregnant rats; forced swimming significantly increased ACTH secretion in both groups compared with values before the swim and basal values, reaching a maximum at 5 min after the swim (Fig. 2a). The ACTH response to forced swimming was significantly lower in pregnant rats compared with that in virgin controls (Fig. 2a), and in the virgin rats the plasma ACTH concentration was significantly less 60 min after the forced swimming than at the peak response, whereas there was no significant decrease in the pregnant–vehicle group (Fig. 2a). Naloxone alone had no significant effect on ACTH concentrations within 15 min after administration in either virgin or pregnant rats (Fig. 2a). However, naloxone significantly attenuated the secretory response to swimming in virgins and there was a significant difference between the virgin vehicle-treated group and all other groups at 5 min after the stress stimulus; the plasma ACTH concentration in naloxone-treated pregnant rats did not differ from that in vehicle-treated pregnant rats (Fig. 2a). The increase in plasma ACTH 5 min after forced swimming, calculated as the difference from the mean basal concentration, was 245 ± 46 pg/ml in virgin rats and the increases in vehicle-treated pregnant (118 ± 20 pg/ml) and naloxone-treated virgin and pregnant rats (154 ± 30 pg/ml, 151 ± 16 pg/ml respectively) were significantly less (one-way ANOVA, $P<0·05$). Naloxone did not significantly affect the plasma concentration of ACTH at 60 min after the swim. Further analysis showed that the effect of naloxone on the stress response was significantly different ($P<0·05$) between virgin and pregnant rats (*t*-test on the difference between the plasma ACTH concentration in the naloxone- and vehicle-treated groups after forced swimming and the calculated S.E.M. of the difference between the two independent means: virgins − 103 ± 58 pg/ml; pregnant +40 ± 29 pg/ml).

Corticosterone Two-way ANOVA for repeated measures of the plasma corticosterone concentrations in all groups showed a significant interaction between time and group ($P<0·0001$); all other specific comparisons were derived from *post hoc* tests ($P<0·05$), unless stated otherwise. Basal plasma concentrations of corticosterone did not differ between virgin and day 21 pregnant rats, and forced swimming significantly increased corticosterone secretion compared with values before the swim and basal values in all groups (Fig. 2b); the corticosterone secretory response to forced swimming in pregnant rats was significantly less than that in virgin rats (at 5 min after the swim only; Fig. 2b). There were no differences among the groups at 15 min after the swim, although concentrations remained similar to those at 5 min within groups. Sixty minutes after the forced swimming, in the pregnant rats the plasma corticosterone concentration had not decreased from the high concentrations at either 5 or 15 min, but that in the virgin rats had returned to basal values (Fig. 2b). Naloxone had no significant effect on basal corticosterone concentration compared with vehicle within 15 min after administration and did not significantly affect the secretory response to stress in virgins (Fig. 2b); however, the corticosterone concentration 5 min after swimming in pregnant rats treated with naloxone was significantly greater than that in the vehicle-treated pregnant rats (Fig. 2b). The increments in plasma corticosterone 5 and 15 min after forced swimming, compared with the mean basal concentration, did not show any significant differences (one-way ANOVA, $P=0·38$, $P=0·94$ respectively, data not shown). Naloxone did not significantly affect the plasma corticosterone concentrations at 60 min after the swim. Further statistical analysis (*t*-test on the difference between the plasma corticosterone concentration in naloxone- and vehicle-treated groups after forced swimming and the calculated S.E.M. of the difference between the two independent means) showed that the effect of naloxone on the stress response was significantly different ($P<0·05$) between virgin and pregnant rats 5 min after stress (virgins − 60 ± 66 ng/ml; pregnant +200 ± 62 ng/ml).

Discussion

We have shown that the oxytocin secretory response to forced swimming (a combined physical and emotional stressor, Abel 1994) persists into late pregnancy, with no differences in plasma concentrations between pregnant

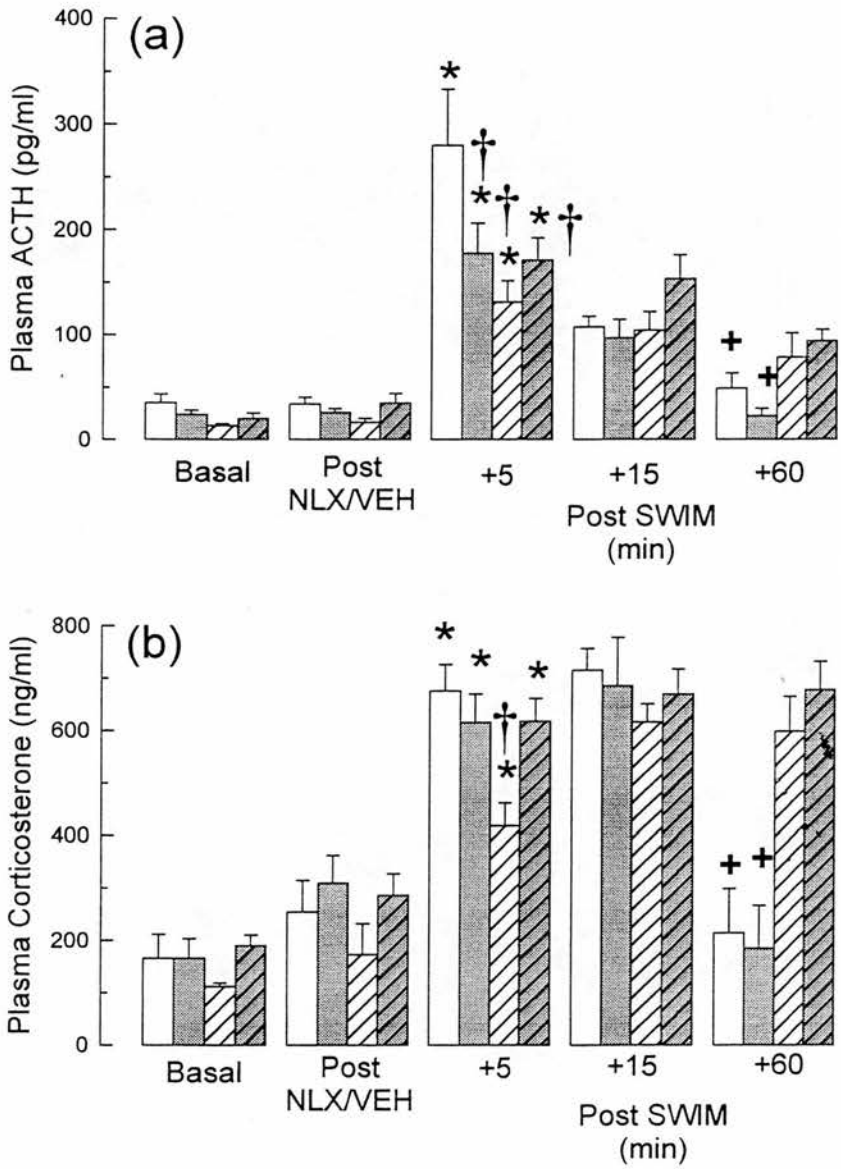

Figure 2 Effect of naloxone on HPA axis secretory responses to forced swimming in pregnancy in the same groups of rats as in Fig. 1. Data are mean ± S.E.M. plasma ACTH (a) or corticosterone (b) concentration in vehicle-treated virgin rats (□) and 21-day-pregnant rats (cross-hatched columns), and naloxone-treated virgin rats (stippled columns) and 21-day-pregnant rats (stippled, cross-hatched columns). Two blood samples were taken before vehicle or naloxone treatment (Basal is the mean of these), further blood samples were taken 5 and 15 min after treatment (post NLX/VEH is the mean of these) and 5, 15 and 60 min after forced swimming (Post SWIM). (a) Newman-Keuls *post hoc* tests, *$P<0{\cdot}05$ compared with before swim; †$P<0{\cdot}05$ compared with virgin vehicle-treated group at same time point; +$P<0{\cdot}05$ compared with peak secretion at 5 min after swim in same animals. (b) Newman-Keuls *post hoc* tests, *$P<0{\cdot}05$ compared with before swim; †$P<0{\cdot}05$ compared with virgin vehicle-treated group at same time point; +$P<0{\cdot}05$ compared with same groups at 5 and 15 min.

and virgin rats (see also Neumann *et al.* 1988); this contrasts with lactation, when the oxytocin secretory response to a stressor is greatly reduced (Carter & Lightman 1987*b*, Higuchi *et al.* 1991, Neumann *et al.* 1995). Oxytocin secretion in response to forced swimming in pregnancy was strongly enhanced by naloxone, which thus indicates that endogenous opioids actually mask an exaggerated response after exposure to this stressor. The lack of a vasopressin response to the forced swim stress used in our studies is consistent with previous reports of findings in male and female rats (Lang *et al.* 1983, Kasting 1988, Wotjak *et al.* 1996), and shows a highly selective activation of the neurohypophysial oxytocin system by the swim stressor. Oxytocin secretory responses to stress, therefore, are restrained by endogenous opioids during pregnancy. Endogenous opioids have previously been demonstrated to inhibit oxytocin, but not vasopressin, secretory responses to immobilisation stress in male (Samson *et al.* 1985) and female virgin rats (Carter *et al.* 1986), and we now show similar endogenous opioid inhibition of oxytocin responses to forced swimming in female rats. Both μ- and κ-opioid systems may be responsible (Carter & Lightman 1987*a*). Endogenous opioids are co-localised and co-secreted with oxytocin and vasopressin (Watson *et al.* 1982, Meister *et al.* 1990) and there is substantial evidence that endogenous κ-opioids restrain stimulated oxytocin secretion at the level of the neurosecretory terminals in the neurohypophysis (Bicknell & Leng 1982). However, previous studies have indicated that, at the level of the neurohypophysis, endogenous κ-opioid inhibitory mechanisms are down-regulated at the end of pregnancy (Sumner *et al.* 1992, Douglas *et al.* 1993), suggesting a greater role for μ-opioids, which act centrally and not at the neurohypophysis (Russell *et al.* 1993).

It is clear that endogenous μ-opioids strongly inhibit oxytocin neurone activity and secretion in late pregnancy (Douglas *et al.* 1995), and that this is manifest mainly on oxytocin neurone cell bodies and their inputs, rather than on the nerve terminals in the neurohypophysis (Douglas *et al.* 1993). The present study has demonstrated that an endogenous opioid mechanism not only restrains oxytocin secretion from an expanded neurohypophysis store in pregnancy (Douglas *et al.* 1993), but also strongly restricts the oxytocin neurone response to forced swimming in pregnancy, as with responses to other stimuli, such as peripheral administration of cholecystokinin during gestation (Douglas *et al.* 1995), and to birth (Hartman *et al.* 1986, Leng *et al.* 1987, 1988, Lawrence *et al.* 1992). This action could be on oxytocin cell bodies themselves, as they have opioid receptors (Inenaga *et al.* 1994, Sumner *et al.* 1992), or on the input pathways to these neurones mediating the stress stimulus, perhaps from the brainstem (Onaka *et al.* 1995). However, endogenous opioids are not responsible for the reduced responsiveness of oxytocin neurones to osmotic stimulation in pregnancy (Bull & Russell 1992), or to electrical stimulation of lamina terminalis (Bull *et al.* 1994) and thus there is likely to be a selective action of opioids on inputs to oxytocin neurones. No consistent changes have been described in magnocellular or parvocellular neurone prodynorphin or proenkephalin A mRNA expression, which are indicators of opioid synthesis, in pregnancy (Schriefer 1991, Douglas & Russell 1994, Douglas *et al.* 1993). However, an increased hypothalamic content of β-endorphin (Wardlaw & Frantz 1983, Dondi *et al.* 1991, Broad *et al.* 1993) and pro-opiomelanocortin mRNA in the arcuate nucleus (Redmond *et al.* 1996) have been described before parturition, which may account for the central endogenous opioid influence on oxytocin neurones in pregnancy.

In parturition, the increased secretion of oxytocin is reduced by the stress of environmental disturbance and the intervals between pup births increase (Leng *et al.* 1987, 1988). The evident dichotomy between these reports and the stimulation of oxytocin secretion by stressors in virgin female and male rats in other previous studies (Lang *et al.* 1983, Gibbs 1986, Carter & Lightman 1987*b*, Wotjak *et al.* 1996) and in the present study on pregnant rats, may be apparent rather than real. Thus the effects of a stressor on oxytocin secretion in parturition could be secondary to the slowing of parturition through another mechanism, with consequent reduced positive feedback stimulation of oxytocin secretion. The effect of naloxone to increase oxytocin secretion in these environmentally disturbed rats (Leng *et al.* 1987) may simply reveal the underlying stimulatory effect of environmental stress on oxytocin secretion as in pregnancy.

This study confirms that secretion of ACTH and corticosterone in response to a stressor are reduced during late pregnancy (Neumann *et al.* 1998) and this is comparable to the reduced HPA axis response to stressors previously reported in lactation (e.g. Walker *et al.* 1995). Naloxone attenuated the increase in ACTH concentration in virgins in response to forced swimming, revealing that endogenous opioids enhance ACTH secretory responses. Naloxone did not attenuate ACTH secretion in response to forced swimming in pregnant rats, indicating loss of the endogenous opioid-enhancing effects on the ACTH secretory response seen in virgin rats. Corticosterone responses showed a trend similar to those of the ACTH responses: there was a pregnancy-related attenuation in corticosterone concentration 5 min after forced swimming and naloxone reversed this; also, high ACTH and corticosterone concentrations after the swim were prolonged in pregnant rats compared with those in virgin rats. However, naloxone did not cause a decrease in the corticosterone response to forced swimming in virgin or pregnant rats at either 5 or 15 min after the swim, although the corticosterone response in virgin rats was not significantly greater than that in virgin or pregnant rats given naloxone. This is in contrast with the response of ACTH, perhaps because the maximal response of the adrenal cortex to ACTH is limiting (Keller-Wood *et al.* 1984). In addition, adrenal

sensitivity to ACTH is increased in pregnancy (Carr *et al.* 1981, Dupouy *et al.* 1975, Waddell & Atkinson 1994), and therefore changes in corticosterone concentration will not necessarily parallel those of ACTH.

We have previously shown that, in pregnancy, down-regulated anterior pituitary mechanisms contribute to the attenuated HPA axis responses, as there is a reduced pituitary ACTH secretory response to exogenous corticotrophin-releasing hormone (CRH) *in vivo* (Neumann *et al.* 1998), attenuated cAMP production in response to CRH *in vitro* and decreased CRH receptor binding (Johnstone *et al.* 1997). We have now shown that, in pregnancy, the enhancing action of endogenous opioid on ACTH release in response to forced swimming, which is normally seen in virgins, is removed. The reduction in ACTH secretion in response to the stressor after naloxone in virgins is consistent with the findings of previous studies showing that opioid antagonists reduce ACTH and corticosterone secretion in response to a stressor in male rats (degli Uberti *et al.* 1995). Naloxone is likely to be exerting its effects on the HPA axis via the hypothalamus (Wang *et al.* 1996), and thus may affect hypothalamic–pituitary mechanisms through CRH release. μ-Opioids appear to mediate the naloxone-induced reduction in stress responses (Cover & Buckingham 1989), whereas κ- and δ-opioids may modulate HPA axis hormone secretion under basal conditions (Iyengar *et al.* 1986, Plotsky 1986). The κ-opioid effects probably occur within both the hypothalamus (Nikolarakis *et al.* 1987) and the anterior pituitary (Calogero 1996). We are not aware of any direct action of naloxone on the adrenal cortex.

The removal of endogenous opioid enhancement on central mechanisms regulating HPA axis responses to stressors in pregnancy may partly underlie the reduced activation of parvocellular PVN neurones by immobilisation (da Costa *et al.* 1996) and decreased CRH mRNA expression in the PVN (Douglas & Russell 1994). Together, these changes constitute evidence for reduced feed-forward activity in the hypothalamo–pituitary component of the HPA axis in pregnancy. The prolonged increase in ACTH and corticosteroid secretion after stress in the pregnant rats is evidently not consistent with enhanced fast negative feedback in pregnancy, but is consistent with either a prolonged adrenocortical secretory response or reduced metabolic clearance, perhaps as a result of increased circulating corticosteroid binding globulin in pregnancy (Seal & Doe 1967). Maternal plasma corticosterone concentrations may be additionally contributed to by the fetus (Dupouy *et al.* 1975). In addition, increased oxytocin secretion after the swimming stress may enhance the secretion of ACTH or corticosterone, or both, by actions on the corticotrophs or adrenal cortex (Samson & Schell 1995, Stachowiak *et al.* 1995, Link *et al.* 1993).

The placenta limits the exposure of the fetus to high concentrations of maternal HPA axis hormones, as ACTH does not cross from the maternal to the fetal circulation (Dupouy *et al.* 1980) and the transfer of glucocorticoid is regulated by several placental enzymes, of which 11β hydroxysteroid dehydrogenase Type II (Seckl *et al.* 1995) predominates in the last few days of pregnancy, inactivating corticosterone (Burton & Waddell 1994). Thus the reduced immediate peak ACTH secretory response to the stressor in pregnancy may act in concert with placental mechanisms to protect the fetus from excessive concentrations of corticosteroid toward the end of pregnancy which could have potentially lifelong deleterious effects (Weinstock 1997). Possible changes in feedback mechanisms in pregnancy are currently under further investigation.

In conclusion, oxytocin neurone secretory responses to exposure to forced swimming are not reduced, but are instead strongly restrained from responding in an exaggerated fashion, by endogenous opioids in late pregnancy. This opioid restraint will contribute to conservation of the neurohypophysial store of oxytocin for primary use in promoting uterine contractions in parturition, when about a third of the oxytocin content is depleted within about 2 h (Fuchs & Saito 1971). HPA axis secretion in pregnancy in response to forced swimming is no longer stimulated by endogenous opioids, which may contribute to protecting the fetus from exposure to excessive concentrations of corticosteroid in the mother during initial responses to stress.

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490.7

EXPRESSION OF GLUCOCORTICOID (GR) AND MINERALOCORTICOID (MR) RECEPTORS IN THE DEVELOPING RAT BRAIN. C. R. Neal, Jr.*, D. M. Vázquez and S. J. Watson, Jr. Mental Health Research Institute, University of Michigan Medical School, Ann Arbor, Michigan 48109-0720.

In addition to the stress of severe illness, premature human neonates may be exposed to glucocorticoid treatment in utero and as neonates. Little is known about the influence of exogenous steroids and stress on normal development of the LHPA axis. In order to better study such effects, this study was undertaken to describe the normal expression of GR and MR in the rat brain at various developmental stages. Pregnant rats and postnatal pups were perfused with Zamboni's fixative. Embryos (E11-E14), fetal brains (E16-E22) and postnatal brains (P1-P21) were post-fixed for 5 days, then soaked in 10% sucrose, frozen at -40°C and cut into 15µm sections. A 347bp fragment of the 3'UT common coding region of MR, a 105bp fragment of the γMR exon and a 500bp fragment of the αGR exon were used for *in situ* hybridization. GR mRNA was detected caudal in mesencephalic epithelium at E14, in hippocampal neuroepithelium at E17, in hypothalamus and cortical plate by E18, and cortex and amygdala by E22. Signal increased in hippocampus and PVN to P14 before approaching adult patterns. MR was first detected in mesencephalic epithelium at E14, cortical neuroepithelium at E18, and hippocampus and amygdala at E22. Signal was low in PVN up to P3, then decreased to adult. Cortical signal was stronger in rostral regions. γMR mRNA signal mirrored that of MR anatomically, but with decreased signal intensity in most regions except cortex and hippocampus where it was similar. These findings demonstrate an increase in GR and MR mRNA expression in the CNS from E14 to P14, which is greater than that seen in the adult. γMR appears to account for a majority of MR expression during development. Immunocytochemical studies of the MR and GR receptors will be performed to correlate these findings. These findings suggest that early steroid treatment may effect brain development. Supported by NIMH Grant 2T32MH15794-17.

490.8

PRENATAL PROGRAMMING OF HIPPOCAMPAL MR AND GR GENE EXPRESSION BY INHIBITION OF 11β-HYDROXYSTEROID DEHYDROGENASE (11β-HSD)

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Prenatal exposure to corticosteroids has been postulated permanently to program hypothalamic-pituitary-adrenal activity. Normally the fetus is protected from maternal glucocorticoids by feto-placental 11β-HSD (type 2), which converts corticosterone into inactive 11-dehydrocorticosterone. This study investigated the effect of inhibition of feto-placental 11β-HSD using carbenoxolone (CBX) on subsequent hippocampal MR and GR gene expression. Female Wistar rats were injected subcutaneously with CBX (12.5 mg/day) or vehicle throughout pregnancy, or were left undisturbed. The CBX injections reduced offspring birthweight by 13%, whereas littersize was not affected. At 12 weeks of age, offspring of CBX-treated rats showed reduced GR gene expression in CA2 (14%) and CA4 (19%) compared to vehicle-treated offspring as measured by *in situ* hybridisation. MR mRNA expression was reduced in dentate gyrus (13%), CA1 (14%) and CA2 (11%). These results were similar to the effects of prenatal dexamethasone treatment[1]. The onset of reduced hippocampal MR and GR gene expression was not immediate and in fact at postnatal day 7 MR mRNA expression was higher in CBX-offspring, specifically in dentate gyrus. Inhibition of 11β-HSD prenatally programmes reduced hippocampal MR and GR gene expression, probably by allowing increased access of glucocorticoids to the fetal circulation.

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490.9

CHANGES IN 11β-HYDROXYSTEROID DEHYDROGENASE TYPE 1 (11β-HSD 1) ACTIVITY AND GLUCOCORTICOID RECEPTOR mRNA IN THE PREGNANT RAT HIPPOCAMPUS AND PARAVENTRICULAR NUCLEUS (PVN). [1,2]H.A. Johnstone, [1]A.J. Douglas, [2]E.A. Antoni*, [1]J.A. Russell & [2]J.R. Seckl
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The neuroendocrine stress response of the hypothalamic-pituitary-adrenal (HPA) axis and PVN CRH mRNA expression are decreased on day 21 of pregnancy (Neumann *et al.* 1996; Douglas & Russell, 1994). To seek evidence for increased glucocorticoid negative feedback on the HPA axis we measured activity of the glucocorticoid-metabolising enzyme 11β-HSD 1 in specific brain regions in pregnant and virgin rats. We also used quantitative in situ hybridisation to measure mRNAs for glucocorticoid (GR) and mineralocorticoid (MR) receptors in the hippocampus and the PVN. 11β-HSD 1 activity in the hippocampus did not change during pregnancy, but in the PVN the activity increased by 54% between virgin and day 16 of pregnancy ($p < 0.05$, $n = 4$-5) and further increased by 29% on day 21 ($p < 0.05$, $n = 6$). GR mRNA expression in the dentate gyrus increased from day 10 to 21 of pregnancy ($p < 0.01$, $n = 5$-6), but did not change in CA1, CA3 & CA4. GR mRNA expression in the PVN did not change significantly but tended to increase in late pregnancy. MR mRNA expression was not altered with pregnancy. Increased 11β-HSD reductase activity in the PVN is expected to increase local glucocorticoid production. Together with increased GR mRNA expression in the dentate gyrus, which has an indirect input to the PVN, this may enhance negative feedback on the HPA axis resulting in suppressed forward drive to the axis.

H.A.J is a BBSRC research student

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2. Douglas A.J. & Russell J.A. (1994). Gene Therapy 1, S85.

490.10

ACUTE BLOCKADE OF CORTICOSTEROID (CORT) RECEPTORS DURING RESTRAINT STRESS IMPAIRS THE EXPRESSION OF HABITUATION TO THE STRESSOR WHEN MEASURING PLASMA CORT, BUT NOT WHEN MEASURING PVN C-FOS INDUCTION IN RATS M.A. Cole*, M.S. Chi, B.A. Kalman, P.J. Kim, R.L. Spencer. Dept. of Psychology, Univ. Colorado, Boulder, CO 80309.

We investigated the interaction of CORT negative feedback with the expression of habituation of the HPA axis response to repeated stress. Male, Sprague-Dawley rats were restrained 1 hr a day in Plexiglas tubes for 6 consecutive days. On day 6, 1 hr prior to restraint stress, rats were injected s.c. with a combination of the selective Type I (RU28318, 50mg/kg) and Type II (RU40555, 30 mg/kg) CORT receptor antagonists or vehicle. In 3 prior studies using this same procedure, the expression of habituation, which was marked by a significant decrease in plasma CORT, was significantly reversed by the day 6 antagonist treatment. The present study examined the concurrent effect of this CORT receptor antagonism on c-Fos protein induction in the PVN of the hypothalamus. Six groups (n=6) were tested: 1) Vehicle + No Restraint, 2) Antagonist + No Restraint, 3) Vehicle + Restraint, 4) Antagonist + Restraint, 5) Vehicle + Restraint (with 5 prior restraint sessions), 6) Antagonist + Restraint (with 5 prior restraint sessions). C-Fos immunoreactivity was visualized on brain sections using immunohistochemistry (primary Ab: rabbit anti C-Fos, Santa Cruz-052). Cell counts indicate that the habituation of PVN c-Fos induction to repeated restraint stress is not reversed by the acute antagonists. This result suggests that the habituation expression reversal for CORT secretion by the antagonists is a consequence of mechanisms that do not involve PVN c-Fos induction. Possible mechanisms include 1) decreased inhibitory or increased excitatory inputs to the PVN not involving PVN c-Fos induction or 2) CORT dependent mechanisms within the PVN not involving c-Fos induction (supported by DK49143; RU28318 and RU40555 were kindly provided by Roussel Uclaf, Romainville, France).

490.11

CHARACTERIZATION OF *IN VIVO* BRAIN MINERALOCORTICOID RECEPTOR (MR) REGULATION USING WHOLE-CELL WESTERN BLOT. B.A. Kalman*, P.J. Kim, M.A. Cole, J.P. Herman, R.L. Spencer. Behavioral Neuroscience Div., Dept. of Psychology, University of Colorado, Boulder, CO 80309 & Dept. of Anatomy, University of Kentucky, Lexington, KY 40536.

We have used an anti-MR antibody (MR 214; a polyclonal antibody directed against an MR fusion protein pGEX) in the western blot procedure to characterize the regulation of MR in whole-cell hippocampal homogenates. This procedure has an advantage over binding studies in that circulating steroids need not be removed before sacrifice, thus avoiding potential re-regulation of receptors. In a study where male Sprague-Dawley rats were killed 24 hr following sham surgery or adrenalectomy (ADX) or 5 days following ADX, a difference in the time-course of regulation was observed between MR and glucocorticoid receptors (GR). While the majority of up-regulation of GR occurred between 1 and 5 days following ADX, the majority of MR upregulation occurred within 24 hr of ADX. This difference does not appear to be due to a difference in cellular compartmentalization of receptors based on comparisons of whole-cell v. cytosolic homogenates. Consistent with binding data, we also found that 5-day treatment with corticosterone (200 mg pellet) but not the GR-specific agonist RU-28362 (10 µg/hr) was able to block the ADX-induced upregulation of MR. These findings suggest that hippocampal MR is regulated by corticosterone and upregulates more rapidly than GR. We wish to thank Dr. Stanley J. Watson for his kind gift of MR 214 antibody (supported by grants, DK49143 & AG12962).

490.12

VALIDATION OF THE *IN VIVO* USE OF RU28318 AND RU40555 FOR ANTAGONISM OF MINERALOCORTICOID RECEPTORS (MR) AND GLUCOCORTICOID RECEPTORS (GR), RESPECTIVELY. P.J. Kim*, B.A. Kalman, M.A. Cole, R.L. Spencer. Dept. of Psychology, Univ. of Colorado, Boulder, CO 80309.

This study examined the suitability of using RU28318 (putative MR antagonist) and RU40555 (putative GR antagonist) to selectively block *in vivo* corticosteroid actions mediated by MR and GR. *In vivo* occupancy of MR and GR by antagonists was inferred from studies examining the dose/time response effects of antagonist treatment of adrenalectomized rats on available cytosolic MR and GR binding in the hippocampus. In addition, studies were conducted to test the ability of the antagonists to block an effect of a MR or GR agonist. We found that RU28318 (50mg/kg, sc) selectively occupied MR in the hippocampus. Further, RU28318 treatment showed no significant agonist effects, however RU28318 was able to block the suppressive effects of aldosterone (120µg/kg, sc) on saline intake in adrenalectomized rats. RU40555 (30mg/kg, sc) selectively occupied GR in the hippocampus. Further, RU40555 showed no significant agonist properties, however RU40555 was able to block the suppressive effects of dexamethasone (50µg/kg, sc) on stress-induced corticosterone secretion. Thus, RU28318 appears to be an effective MR antagonist and RU40555 appears to be an effective GR antagonist *in vivo*. [Supported by UROP program at the Univ. of CO, and USPHS grants DK-49143 and MH54742. RU28318 and RU40555 were kindly provided by Roussel Uclaf, Romainville, France]

P191 ^{125}I-oCRH BINDING AND CRH STIMULATION OF cAMP PRODUCTION BY THE RAT ANTERIOR PITUITARY *IN VITRO* ARE REDUCED IN LATE PREGNANCY

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The hypothalamic-pituitary-adrenal axis response to stressors is attenuated in late pregnancy, and CRH is less effective in stimulating ACTH secretion *in vivo* (Neumann *et al.* 1996). So we have now measured basal and CRH-stimulated levels of cAMP, the second messenger for the pituitary CRF-1 receptor ($CRHR_1$), in anterior pituitaries from day 20 pregnant and virgin rats, and receptor binding using ^{125}I-oCRH on fresh frozen pituitary sections. Animals were decapitated and the anterior pituitaries cut into eight segments and incubated in the presence or absence of 10nM CRH for 10 mins. The tissues were then triturated in ice-cold 0.2N HCl for analysis by cAMP RIA. For $CRHR_1$ autoradiography, 20µm sections were incubated with ^{125}I-oCRH in the absence (total binding) or presence (non-specific binding) of excess cold oCRH. Film was exposed for 3 weeks and the silver grain density measured with an image analyser. CRH increased cAMP content less in pituitaries from day 20 pregnant rats than in virgins (315 ± 34 vs. 463 ± 43 pmol/50µl ± sem, $p < 0.01$., $n = 8$). $CRHR_1$ autoradiography showed less binding in the anterior pituitary from day 21 pregnant vs. virgin rats (0.21 ± 0.04 vs.0.52 ± 0.04 grain density, $p < 0.01$). Grain density was also reduced in the intermediate lobe in pregnancy. The results indicate reduced density of $CRHR_1$ in the anterior pituitary in late pregnant rats, leading to a reduced cAMP response to CRH, and thus to a reduced ACTH response to stressors *in vivo* at the end of pregnancy.

References: Neumann I., Johnstone H., Landgraf R., Russell JA. & Douglas AJ. (1996). *J. Endocrinol.* **148,** suppl. P131. (HAJ is a BBSRC research student. Supported by a British Council/DAAD ARC grant).

P192 DOES THE EXCESS CANCER INCIDENCE AND MORTALITY IN ACROMEGALY HAVE A GENETIC BASIS?

S M Orme, J Crockford*, D T Bishop* and P E Belchetz ; Department of Endocrinology, The General Infirmary at Leeds and *Imperial Cancer Research Fund Epidemiology Centre, Leeds LS1 3EX.

We have previously reported an excess overall cancer mortality in acromegaly (O/E ratio 1.33 p=0.001), with an excess colon cancer incidence (O/E 1.98 p=0.022) and mortality (O/E 2.8 p=0.001). This study suggested a relationship between cancer mortality and post treatment growth hormone (GH) levels in acromegaly.

However, if there is a genetic predisposition (such as the loss of a tumour suppressor gene) to develop cancer in certain acromegalics, which may not be related to the degree of GH hypersecretion, then this cancer excess may also be reflected in an increased cancer incidence and mortality in the relatives of acromegalics.

We therefore investigated the first degree relatives of 30 acromegalics (257 subjects; 32 acromegalics, 60 parents, 93 siblings and 72 children). We compared cancer rates between the families studied and data from the Yorkshire Cancer Registry, by life table analysis.

There was no increase in all cause cancer rate or colon cancer rate (O/E 1.047 p=0.476) in the first degree relatives of these acromegalics.

In conclusion these data do not support the hypothesis that cancer excess and particularly colon cancer excess in acromegaly has a genetic basis.

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period using an unpaired t test. Electrophysiological recordings were obtained from thirty arcuate neurones (mean firing rate: $0{\cdot}74 \pm 0{\cdot}14$ spikes s^{-1}, mean ± S.E.M.) that projected to the median eminence. The activity of eighteen of these cells was unaffected by injection of MK-0677 ($0{\cdot}76 \pm 0{\cdot}10$ to $0{\cdot}72 \pm 0{\cdot}10$ spikes s^{-1}, $n = 8$) or GHRP-6 ($0{\cdot}43 \pm 0{\cdot}10$ to $0{\cdot}48 \pm 0{\cdot}11$ spikes s^{-1}, $n = 10$). However, the activity of five arcuate neurones was significantly increased following injection of GHRP-6 ($0{\cdot}80 \pm 0{\cdot}16$ to $1{\cdot}85 \pm 0{\cdot}2$ spikes s^{-1}, $P < 0{\cdot}01$) and a further seven cells were excited by injection of MK-0677 ($0{\cdot}95 \pm 0{\cdot}12$ to $1{\cdot}63 \pm 0{\cdot}16$ spikes s^{-1}, $P < 0{\cdot}01$). Both drugs significantly elevated the mean firing rate at 10 min after injection and the firing rates remained elevated for the subsequent 30 min. Cells excited by either GHRP-6 or MK-0677 could not be subsequently activated by a second injection (at least 40 min after the initial injection) of the same secretagogue, negating the possibility of examining the actions of both secretagogues on the activity of single neurones. These results suggest that the orally active GH secretagogue, MK-0677, activates a subpopulation of neuroendocrine arcuate neurones in a similar manner to GHRP-6 and may therefore prove a useful tool in investigating the central control of GH release.

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Changes in 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD 1) activity in the rat hypothalamic paraventricular nucleus (PVN) and anterior pituitary during pregnancy

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At the end of pregnancy (day 21) corticotropin-releasing hormone (CRH) mRNA expression is significantly decreased in the PVN (Douglas & Russell, 1994), consistent with attenuation of the neuroendocrine stress response in late pregnancy (Neumann *et al.* 1996). This may reflect an increased glucocorticoid negative feedback on the hypothalamic–pituitary–adrenal (HPA) axis at the level of the PVN. 11β-HSD 1 is a NADPH-dependent enzyme which, *in vitro*, reversibly metabolizes glucocorticoids to their inactive metabolites (Moisan *et al.* 1990), thus regulating their access to both mineralocorticoid (MR) and glucocorticoid (GR) receptors, but *in vivo* it can also activate glucocorticoids (Rajan *et al.* 1996). Altered activity of 11β-HSD 1 in the hypothalamus could thus regulate feedback onto CRH neurones.

We have measured 11β-HSD 1 activity in the brain in day 16 and day 21 pregnant rats and in virgin controls, which were decapitated for tissue harvest. Micropunched PVN from vibroslice brain sections, dissected hippocampus and cerebellum and the anterior pituitary were homogenized, then incubated at a protein concentration of either 200 or 500 μg ml^{-1} with 200 mM $NADP^+$ and 12 nM [1,2,6,7 ^{3}H]corticosterone (specific activity 82 Ci $mmol^{-1}$) for 60 min at 37 °C. Each PVN determination was made on micropunches from two rats. The steroids were then extracted into ethyl acetate, dried down, reconstituted and run on TLC plates. 11β-HSD activity was expressed as percentage conversion of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone.

We found that 11β-HSD activity in the hippocampus ($26{\cdot}17 \pm 1{\cdot}77$, $23{\cdot}63 \pm 2{\cdot}40$ and $26{\cdot}52 \pm 1{\cdot}74$%, means ± S.E.M., virgins, day 16 and day 21, respectively), and the cerebellum ($21{\cdot}15 \pm 3{\cdot}27$, $21{\cdot}33 \pm 1{\cdot}19$ and $23{\cdot}51 \pm 1{\cdot}97$%) did not change during pregnancy. In contrast, activity in the anterior pituitary significantly increased between day 16 and day 21 ($16{\cdot}81 \pm 1{\cdot}89$ to $22{\cdot}7 \pm 0{\cdot}97$%, $P < 0{\cdot}05$, Mann–Whitney test, $n = 10$–12 determinations). However, the greatest change occurred in the PVN where activity approximately doubled between virgin controls and day 16 ($4{\cdot}10 \pm 0{\cdot}53$ to $8{\cdot}83 \pm 1{\cdot}17$%, $P < 0{\cdot}05$, $n = 4$–5), with a further increase at day 21 ($12{\cdot}50 \pm 3{\cdot}80$%, $P < 0{\cdot}05$, $n = 6$).

The PVN is central to the activation of the HPA axis in response to stress. It receives inputs from the periphery as well as from other brain areas, and is an important site for the synthesis of several ACTH secretagogues, including CRH. Thus increased 11β-HSD 1 reductase activity in pregnancy may contribute to an enhanced negative feedback by generating glucocorticoids within the PVN and the anterior pituitary.

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